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AN EVALUATION OF THE SENSITIVITY OF MICROASSAY RELATIVE TO TROUT AND DAPHNID ACUTE LETHALITY TESTS

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AN EVALUATION OF THE SENSITIVITY OF MICROASSAYS RELATIVE TO TROUT AND DAPHNID ACUTE LETHALITY TESTS

by

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North Vancouver, British Columbia

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ABSTRACT

PROJECT TITLE: An evaluation of the sensitivity of microassays relative to trout and daphnid acute lethality studies.

CONTRACT NO: SSC File No. XSG88-00021-(602)/A
E.V.S PROJECT NO: 3/064-30
SCIENTIFIC ADVISOR: Mr. Gary Sergy, Environment Canada, Edmonton (403) 468-8039
PERFORMANCE PERIOD: December, 1988 - March, 1989

PROJECT DESCRIPTION:

The purpose of this report was to summarize the data describing correlations between two types of bioassays used for legal purposes (rainbow trout, *Daphnia*) and selected aquatic toxicity microscale tests. The selection process reviewed more than 50 microscale tests which utilized algal, protozoan or bacterial species (summarized in Appendix A). The tests were categorized based on criteria describing portability, time required, ease of use, cost and complexity. The tests selected for the highest applicability to the field, were the Microtox, Toxichrome, Resazurin reduction, ECHA biocide monitor, ATP-TOX and SOS-Chromotest, all of which are available commercially.

Limitations of the data set were that (1) any summary of relative sensitivity varied with the compounds and organisms tested; (2) there was a lack of standardization in approach and a failure to provide adequate details on the origin of the cited toxicity data which confused and obscured comparisons; and (3) published reviews commonly failed to identify fish species and test type for cited data. Most of the data available for comparison related to the Microtox test, and correlations were separated based on chemical groups of organics, inorganics, municipal waste, petroleum wastes, industrial effluents and sediment extracts or leachates. Correlations of Microtox with "legal" bioassays were described by chemical group. Very little information was available for other microtests, and the data were summarized accordingly.

For field use of microassays, the best approach would involve a battery of several tests, with the final test selection dependent upon *a priori* knowledge of the gross characteristics of the contamination involved. Appropriate microassays could be selected for sample screening, based on present information about the relative sensitivities of tests to different chemical groups. However, there are data gaps where information is not sufficient to select an appropriate microassay; in these situations *Daphnia* or rainbow trout bioassays could be used.

RÉSUMÉ

TITRE DU PROJET: Évaluation de la sensibilité de microbioessais pour les études de toxicité aiguë chez la truite et les daphnées.

Nº DU CONTRAT: Dossier SSC Nº XSG88-00021-(602)/A

PROJET E.V.S. Nº: 3/064-30

CONSEILLER SCIENTIFIQUES: M. Gary Sergy, Environnement Canada, Edmonton (403) 468-8039

PÉRIODE DE RENDEMENT: Décembre 1988 - mars 1989

DESCRIPTION DU PROJET:

Le but du présent rapport est de résumer les données décrivant des corrélations entre deux types de bioessais utilisés à des fins légales (truite arc-en-ciel, Daphnia) et des essais choisis de toxicité aquatique à l'échelle micro. Le processus de sélection a étudié plus de 50 essais à l'échelle micro qui utilisaient des espèces d'algues, de protozaires ou de bactéries (résumé dans l'Annexe A). Ces essais ont été répartis par catégorie d'après des critères décrivant leur portabilité, le temps qu'ils nécessitent, leur convivialité, leur coût et leur complexité. Les essais choisis parce qu'ils étaient plus facilement applicables sur le terrain étaient les essais Microtox, Toxichrome, de réduction Resazurin, de surveillance des biocides ECHA, ATP-TOX et SOS-Chromotest, qui sont tous disponibles dans le commerce.

Les limites des ensembles de données étaient les suivantes: 1) tout résumé de la sensibilité relative variait selon les composés et les organismes testés; 2) on a noté un manque de standardisation dans l'approche et l'insuffisance des précisions concernant l'origine des données de toxicité mentionnées, ce qui est à l'origine de confusion et de maque de clarté quand on fait des comparaisons; et 3) les comptes rendus publiés n'identifient pas les espèches de poissons et les types d'essais correspondants aux données mentionnées. La plupart des données disponibles pour les comparaisons ont été obtenues par l'essai Microtox, les corrélations ont été séparées d'après les groupes chimiques des produits organiques, inorganiques, de déchets municipaux, de déchets de pétrole, d'effluents industriels et d'extraits ou d'eau de percolation de sédiments. Les corrélations d'essais Microtox avec des bioessais de médecine légale ont été décrites pour divers groupes chimiques. Très peu d'informations étaient disponibles pour d'autres micro-essais, et les données ont été résumées en conséquence.

Pour l'utilisation sur le terrain de micro essais, la meilleure approche consiste à utiliser une batterie d'essais, la sélection de l'essai final dépendant d'une connaissance a priori des caractéristiques générales de la contamination. Des micro-essais appropriés peuvent être choisis pour le cépistage des échantillons, d'après les informations actuelles sur les sensibilités relatives des essais à différents groupes de produits chimiques. Toutefois, il existe des lacunes au niveau des données, et les informations ne sont pas suffisantes pour choisir un micro-essai approprié; dans ces cas, il faut utiliser des bioessais avec les Daphnia ou la truite arc-en-ciel.

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1.0 INTRODUCTION

Historically, Canada has been a leader in the development and application of biological testing. The rainbow trout static test was one of the first aquatic toxicity tests cited in federal regulations and used effectively in litigation. The most recent advance in test protocol development has been the *Daphnia* acute lethality test published for Ontario (Poirier et al., 1988).

Existing "legal" toxicity tests (bioassays) are based on the assumption that a "most sensitive species" can be used as an indicator of ecosystem responses. However, present "legal" toxicity tests require several days for completion and are costly if large numbers of samples are required to identify the extent of an impacted area and/or evaluate large numbers of chemicals or effluents.

"Legal" tests do, however, offer the advantages of producing results which can be understood by the public as well as evidence that has some validity in law, both of which are important aspects not likely to be changed easily or quickly. Pressure to conduct biotesting is increasing due to legislation and regulation such as the Canadian Environmental Protection Act (CEPA) and the Ontario Municipal and Industrial Strategy for Abatement (MISA).

Environment Canada is reviewing its ecotoxicological procedures, and "second generation" biotests that are small scale and sensitive fill a niche in toxicity assessment (Blaise et al., 1988). These microscale toxicity tests are an important asset as adjuncts to "legal" tests. They have the potential for rapid turn-around times, low costs, compatibility for use in the field and use with multiple organisms.

One specific type of need is for rapid microscale aquatic toxicity tests, the results from which can be used as an indicator of toxicity measured by prescribed "legal" tests such as the rainbow trout and daphnid tests. Uses of such screening type tests are related to:

- mobile ultrafiltration/microfiltration/reverse osmosis water treatment units being developed and demonstrated by RRETC. More specifically, to quickly determine if the processed (clean) waters are no longer deleterious and treatment can cease.

- chemical/oil spill enforcement actions. More specifically, to screen samples and indicate if and where, a "legal" bioassay sample should be collected and a "legal" test performed, e.g., rainbow trout assay.

- compliance monitoring in remote locations. More specifically, to indicate, if a "legal" bioassay sample should be collected, which can be an onerous and costly undertaking.

- tracing of sources of toxicity in open waters or in large sewage systems.

- evaluation of large numbers of small volume samples to assess relative toxicity and to determine which samples require further testing.

A microscale test needs to incorporate as many aspects of toxicological assessment techniques as possible; for example: exposure route, age, life cycles, and acute and chronic (if possible) evaluations. Criteria for suitable microscale test(s) include a relatively quick turn-around time (ideally in a few hours or less); modest space, labour and operator training requirements; portability, for use outside of a permanent laboratory; and sensitivity.

1.1 Objectives

This project describes, for various substances, classes or mixtures of substances, the correlation (or relationship) between:

- selected aquatic toxicity microscale test(s) results; and,
- specified aquatic toxicity tests used for "legal" purposes.

1.2 Approach

A wide variety of bioassays are available which have the potential to be adapted to field usage. To deal with the large amount of available data, this review assessed the applicability of test protocols for field usage (Section 2) and selected those tests most amenable to field testing. Descriptions of the various tests reviewed are found in Appendix A. For those tests which were selected, available data describing the relationships of the microassays to established "legal" tests were summarized.

For the majority of tests, direct relationships have not been investigated, or have only been examined for a few chemical groups. These data have been summarized accordingly.

1.3 Battery vs. Screening Approaches

A battery approach to toxicity testing is used to thoroughly evaluate the toxicity of an effluent, compound or sediment. Several tests are conducted on the same sample which span a range of end points, exposure routes, taxa and lifestyles. Ideally, a battery of bioassays would completely characterize the toxicity of the sample. On the other hand, screening tests are used to identify samples which require further testing. Ideally, they would be sensitive

enough to all chemical groups to screen out samples which are non-toxic.

There are several problems associated with using a single assay for screening samples or monitoring toxicant/contaminant levels. Even within specific tests, the use of different bacterial species can markedly alter the spectrum of sensitivity of the assay. The use of a single bioassay as a screening test increases the chance that a sample will be identified as non-toxic, when it is actually toxic. The main driving force behind the use of a battery of tests is the inconsistency of results obtained when samples are tested in inter-test comparisons. For example, when 156 pollutants were examined by Bringmann and Kuhn (1980), 47 were most toxic to algae, 43 to protozoans and 23 were selectively toxic to bacteria. Similar findings have been reported by Dutka et al. (1988a,b), Williams et al. (1986a,b), Dutka and Gorrie (1989) and Samoiloff (1989) (Table 1). Dutka and Kwan (1988) recommend a battery of tests encompassing 2 to 3 genera and consisting of 2 or 3 toxicant/geno-toxicant assays as well as 2 or 3 microbial hazard screening tests. Their recommendations were based on a series of monitoring tests done over almost a decade of research.

However, the main disadvantage of the battery approach is that several tests must be conducted; this can be expensive and time consuming and likely inappropriate for screening large numbers of samples. If prior knowledge of an area is available, it should be possible to choose one or two microassays based on the specific test sensitivities relative to expected sample characteristics. This review provides information on which microassays will provide data which can be compared to trout or daphnid tests, for different classes of contaminants. There is an enormous literature available for comparison of individual chemicals, especially organics, for some microassays (notably Microtox). This report does not include a full listing of the more than 600 chemicals tested for Microtox (Kaiser and Ribo, 1988), nor does it replicate any graphic figures describing the relationships between microassays and macrobioassays (Ribo and Kaiser, 1983). Comparisons are limited to categorization of groups of chemicals, and tabulation of the available correlation coefficients describing relationships between various assays.

Table 1. Proposed batteries of assays suggested for complete field evaluations by various researchers.

Assays	Reference	Toxicant Source
Daphnia, ATP-TOX, Microtox, SOS-Chromotest, *Spirillum*, Algal ATP	Dutka et al., 1988b	water
Selenastrum capricornutum, Microtox, SOS-Chromotest	Blaise et al., 1988	inorganic and organic toxicants
Bacterial inhibition, Bacterial dehydrogenase, Bacterial oxygen consumption, *Daphnia*, Larval fish lethality, *Legionella*, Caprostanol levels, Cholesterol levels	Dutka and Kwan, 1982	sediments
A1-MPN fecal coliform, *Clostridium perfringens*, SOS-Chromotest, algal ATP, *Daphnia magna*	Dutka, 1988	water-extracts of sediments
Microtox, Algal ATP, SOS-Chromotest	Dutka, 1988	1% DMSO extracts of sediments
Microtox, Oyster embryo test, Amphipod lethality	Williams et al., 1986b	sediments (marine)
Daphnia, Microtox	Giesy et al., 1988	sediments
Chemistry, Nematode lethality, Microtox	Samoiloff, 1989	sediments

1.4 Limitations of Published Microassay Data Sets

Most limitations will be described in association with examples from Microtox studies, solely because most of the papers reviewed dealt with the Microtox assay. However, these observed limitations are consistent for most microassay comparisons.

Many microassay comparisons produce an extensive list of effluents or sediments which were tested, without any specific identification of their constituents. In most cases, the data are summarized either on a qualitative, subjective basis (it was "obvious" that a was more sensitive than b) or as a mean difference in relative sensitivity. The latter calculation results from an estimation of the difference in sensitivity (e.g. Microtox 10 x as sensitive), and is based on the average relative difference in EC50 values. The final average is dependent upon the denominator of the ratio, and can be altered to show that either a or b is more sensitive, using the identical data set. These approaches only provide superficial qualitative data, since the average relative sensitivity of several studies can be greatly influenced by a single test which shows a difference of 1000 or 10000 times in sensitivity between two bioassays. For example, the sensitivity of macroinvertebrates to cadmium varies over 6 orders of magnitude (Williams et al., 1986a), and the inclusion of cadmium in a comparative data set can greatly influence average sensitivity. All measures of relative sensitivity are directly related to the compounds and organisms used in testing for that specific comparison, and should not be interpreted independent of those chemicals and test specifics.

Another approach presents data as a percentage agreement, where the sensitivity of one test is related to another purely on the basis of positive versus negative responses. The major concern with this type of comparison is the necessity for an arbitrary decision of what is toxic and what is non-toxic. Some authors separate toxic and non-toxic responses simply based on a positive response, as evidenced by the EC50. Another approach used by various authors is to separate toxicity into different classes of toxicity (e.g., Bulich, 1982) where an LC/EC50 of less than 1% represents a class I chemical, class II is 1.0 to 3.2 and class III 3.2 to 10% (log scale

increments), and so on. Relative agreements in these cases rank the number of times two tests rank a sample in the same class, although different ranking schemes are used by different researchers. The percentage agreement between the rankings of two tests are then compared or summarized into a single number, which is reported in the literature. At this point, there is no standardized approach and some authors do not outline the procedure used to derive the final number used for comparison.

Another problem with the use of percentage agreement is that different assays can only test a certain maximum concentration of an environmental sample, and this varies widely between tests. Microtox, due to requirements of sample dilution and preparation, can only test an environmental sample to a maximum of 45% of its collected concentration, the SOS-Chromotest to <5% and *Selenastrum* to 80%, whereas daphnid and trout assays can test a sample at 100% of collected sample concentration (Greene, pers. comm.).

The most common misuse of data summaries involves the use of "fish' LC50s for comparative purposes, without an adequate description of the species of fish, the duration of exposure or the source of the original data (e.g., Bulich et al., 1981; Chang et al., 1981; Bulich, 1982; Curtis et al., 1982; Bitton, 1983). These summaries have become entrenched in the literature and are commonly cited as support for a high correlation of microassays (notably Microtox) with fish lethality assays. Tracing to the original data becomes time consuming since most of these comparisons collected their "fish" data from previous summaries which are distant from the original data source. This problem is further complicated by the summary of data into a single number, which is meaningless when taken out of context. For example, De Zwart and Sloof (1983) concluded that fish lethality assays are 1.15 times as sensitive as Microtox, and this paper is frequently cited in other summaries. However, this number derives from an average of the relative sensitivity of four species of fish compared to Microtox (rainbow trout- 2.04; fathead minnow- 1.99; *Poecilia reticulata*- 0.66; and *Oryzias latipes*- 0.65) and was based on a comparison of fish 48 h LC10 to 5 min Microtox EC10 values. The latter qualifications are seldom cited along with the figure of 1.15 x difference in relative sensitivity. The 5 min EC10

underestimates the Microtox EC50 by a factor of >4 (range 1.9 to 7.7; reanalysis of De Zwart and Sloof, 1983). The correlation of fish 48 h LC10s to the more traditional 96 h LC50 is unknown, but would undoubtedly overestimate the LC50 value by a large factor. A re-comparison of the Microtox data De Zwart and Sloof (1983) used to derive their "sensitivity factor" with the original fish data (Sloof et al., 1983) shows that the rainbow trout LC50 is more sensitive than Microtox 15 min EC50 13/15 times, with an average difference in those cases of >118 x (range 1.1 to 1453). Similar changes are seen for the fathead minnow data; the fathead minnow is more sensitive than Microtox 12/15 times, with an average of 15.4 in those cases (range 1.9 to 99.1). Again, the average relative sensitivity values are strongly influenced by the testing of a few chemicals. These estimates of 118 x and 15.4 x are considerably different from the values of 2.04 and 1.99 cited by De Zwart and Sloof (1983), but are probably more realistic.

One of the original descriptions of the relative sensitivity of Microtox and fish assays was provided by Bulich et al. (1981). The paper tabulated the response of Microtox to 20 different chemicals and the relative responses of fish lethality assays. The values quoted for fish are summaries of a range of values for a wide variety of species with tests of varying duration, ranging from 24 to 96 h LC50s. The papers cited as the data source are, for the most part, reviews of data rather than original research papers. Bulich et al. (1981) summarize their tabulated data in a figure which shows the very high comparability of fish and Microtox LC/EC50s. The data set presented is misleading in several ways. For a start, the isopropanol data point is misplaced on the figure by an order of magnitude, and two data points present in the Table are absent in the Figure (formaldehyde and butanol). Furthermore, the tabulated ranges of LC50 values do not include all appropriate minimum values from cited papers. For example, for copper there are many values in papers from Bulich's reference list which yield an LC50 value for copper which is less than 0.070 mg/L, and values for cyanide are recorded (in Bulich's cited literature) as low as 0.012 mg/L. The minimum range in the table for both is 0.1 mg/L. Appropriate minimum values for other chemicals are also missing. However, this does not have a great impact on the authors' interpretation of the data because the values

plotted on the corresponding figure are average values, taken as the mean of the upper and lower LC50s recorded in their references. Summarization of the data in this manner appears to overestimate the toxicity of copper, trinitrotoluene and malathion by a factor greater than 10-fold, and chromium more than 30-fold. An acceptable average LC50 for copper, using references 4, 9 and 11 from the cited list (Bulich et al., 1981), was 0.4 mg/l for 14 bioassays, with a range from 0.060 to 1.42 mg/L, relative to the tabulated average of 5.4 mg/L. For chromium, the range of toxicity (found in references 4, 9 and 11) was from 0.105 to 2.16 mg/L (average 0.78, n=7), relative to the range reported by Bulich (for references 4 to 12) of 29 to 133 mg/L; an overestimation of >50-fold. The inclusion of rare LC50s using exotic species or unorthodox exposure regimes alters the range of values and the "average LC50", and skews the appearance of the relationship. The range of toxicity for benzene cited by Bulich is 17 to 32 mg/L, although a 1 h LC50 for brown trout was only 12 mg/L (in Bulich's cited literature). The inconsistencies in the data set make the relationship between Microtox and fish toxicity less clear than described and repeatedly cited.

The major limitations of the data available for interassay comparisons can be summarized in three main points:

1) **Any summary of relative sensitivity will vary with the identity and number of compounds and organisms tested.**
2) **A lack of standardization in approach and failure to provide adequate details on the origin of the cited toxicity data can confuse and obscure comparisons.**
3) **Generalizations commonly fail to identify fish species and test type for cited data.**

The correlations reviewed in this paper evaluate the comparability of data based on individual chemicals, and cautious generalizations are made on chemical groups which appear to act similarly. Data summaries with the limitations described above did not play a large role in defining relative sensitivities.

2.0 RATING OF MICROASSAYS FOR APPLICABILITY TO THE FIELD

2.1 Categorization of Microscale Assays

The large number of microscale tests required that the tests be divided into general groups before categorization of the applicability of each test to the field. For example, there are many tests describing the utilization of oxygen consumption by bacteria for field assessment, and bacterial enumeration techniques can be conducted for any one of a dozen or more species of bacteria. However, in each of these cases, the applicability to field assessment is the same, although correlations to laboratory assays may be quite different.

The general categories of tests which are available are those examining: a) natural density, b) natural metabolic activity (after substrate addition or enzyme addition) and c) impacts of toxicant on normal metabolic functions (growth, motility, respiration, fluorescence, enzyme activity). In several cases the relationship of bacterial metabolism to toxicant action has been utilized to the level where test kits are commercially available.

To assess applicability to the field, several test characteristics were considered, including sample time, labour and operator training requirements and portability. Factors were also included for complexity of test and interpretation difficulty. Characteristics which were subjectively rated were portability, time, ease of use, complexity, amount of training, interpretation difficulty and cost.

All aspects were subjectively rated as high, medium or low. The characteristics which were most desirable (i.e., high portability, fast sample time, low costs) received a value of 1, while those least desirable received a value of 5. Subjective ratings for each test evaluated are described in detail on Table 2. This subjective rating was necessary to sort through the 50 or so microtests available and identify those tests which were

Table 2. **Prioritization and ranking[1] of the suitability of microassays for the screening of toxicant samples in the field. Refer to Appendix A for descriptions and references for each test.**

Group	Characteristic	Response	Species	Portability	Time	Ease of Use	Complexity	Amount of Training	Interpretation Difficulty	Cost	Rating Total	Rating Avg.
ALGAE												
	enumeration	macroplate counts	various	High	Med.	High	Low	Low	Low	Low	9	1.3
	metabolism	dehydrogenase activity	various	Med.	Fast	Med.	Med.	Low	Low	Med	17	2.4
	growth	ATP content	various	Med.	Fast	Med.	Med.	Med.	Low	Med.	17	2.4
PROTOZOA												
	metabolism	respiration	various	High	Fast	High	Low	Low	Low	Low	7	1.0
	growth	growth inhibition	Colpoda	Low	Slow	High	Med.	Med.	Low	High	23	3.3
BACTERIA												
	enumeration	density	various	Med.	Med.	High	Low	Low	Med.	Low	13	1.9
		coliphage	various	Med.	Slow	High	Low	Low	Low	Low	13	1.9
	metabolism (natural)	sterol levels in sediments	E. coli	Low	Med.	High	Med.	Med.	Med.	Low	19	2.7
		O_2 consumption	various	High	Fast	High	Low	Low	Low	Low	7	1.0
		CO_2 production	E. coli	High	Fast	Med.	Med.	Med.	Low	Low	13	1.9
	growth	density reduction	various	Med.	Med.	High	Low	Low	Med.	Low	13	1.9
		inhibition	Bacillus sp.	High	Med.	High	Low	Low	Low	Low	9	1.3
		agar diffusion	various	High	Med.	High	Low	Low	Low	Low	9	1.3
		ATP changes	various	High	Fast	Med.	High	Med.	Low	Med.	17	2.4
	metabolism (altered)	fluorescence	Photobacterium	High	Fast	High	Low	Low	Low	Med.	9	1.3
		nitrification	Nitrobacter	High	Fast	Med.	Low	Low	Low	Med.	11	1.6
		CO_2 production	E. coli	High	Fast	Med.	Med.	Med.	Low	Low	13	1.9
		motility	Spirillum volutans	Low	Fast	Med.	Low	Med.	Med	Low	17	2.4
		O_2 consumption	various	High	Fast	High	Low	Low	Med.	Low	9	1.3
		ATP levels	activated sludge	High	Fast	Med	High	Med	Low	Med.	17	2.4
		beta-galactosidase activity	E. coli	High	Fast	High	Low	Low	Low	Med.	9	1.3
		SOS-DNA repair	E. coli	Med	Med.	Med.	Med	Low	Low	Med	17	2.4
		dehydrogenase activity	Bacillus cereus	High	Fast	High	Low	Low	Low	Med.	9	1.3

1. Each test was ranked on an interpretation of its suitability for field application based on 7 characteristics. An ideal test would receive a ranking of 7 (average 1.0). Average values between 1 and 1.5 were desirable, 1.5 - 2.0 of possible use and ≥2.0 undesirable.

Portability: High (1): easily applicable to field with generator use
Medium (3): minimal laboratory facilities required
Low (5): not applicable to field

Time: Fast (1): ≤12 h
Med. (3): <24 h
Slow (5): >24 h

Ease of Use: High (1): single person could accomplish after minimal training.
Med. (3): some expertise would be required
Low (5): only a highly trained individual could accomplish

Complexity: Low (1): few procedural steps
Med. (3): many procedural steps
High (5): complex procedural steps

Amount of Training: Low (1): no specialized talents or techniques required
Med. (3): requires some practical experience
High (5): difficult and requires some technical expertise

Interpretation Difficulty: Low (1): results are clear
Med. (3): results are subjective or qualitative
High (5): interpretation may be ambiguous

Cost: Low (1): little initial equipment required
Med. (3): some initial equipment required, but subsequent tests are cheaper
High (5): specialized laboratory facilities required

to be considered for field applicability, and for which comparisons with "legal" tests should be described.

After subjective rating, tests were categorized into one of four groups, based on their perceived value as a field microassay. Tests which were very useful as field microassays were placed in the highest level (category I) (Table 3). Tests which were potentially useful as a field microassay but were indirectly comparable to macro-bioassays or were more complex were identified as category II. Microtests which required some laboratory facilities but could potentially be adapted for field assay conditions were placed in category III. Tests which were restricted to laboratory conditions, or required more than 24 h to complete, were placed in category IV.

2.2 Environmental Relevance

Tests in each category were placed in sub-categories of environmental relevance representing high, medium and low correlation to macrobioassays (Table 3). Tests of high environmental relevance were tests for which data were available describing a relationship between the microscale test and some macrobioassay test. Tests of low environmental relevance showed comparability with some other measure of toxicant behaviour, such as fish opercular rhythms.

Relationships with macrobioassay tests were described for all tests from categories I, II or III with a high environmental relevance. Such tests are those measuring bacterial fluorescence, inhibition, enzyme activity and ATP content (Table 4).

3.0 COMPARISONS OF MICROASSAYS WITH "LEGAL" BIOASSAYS

Tests separated by the selection procedure for consideration as field microassays, included tests involving bacterial fluorescence, inhibition and enzyme activity (category I), bacterial ATP measurements (category

Table 3. Environmental relevance[1] and categorization[2] of microassay tests.

Group	Characteristic	Trade Name	Rating Avg.	Sample Time	Special Equipment Requirement	Advantages	Disadvantages	Environmental Relevance	Category	Reference
ALGAL	growth or enzyme activity		2.4	4 h	spectrophotometer or luminometer	sensitive to herbicides	4 h estimate very crude relative to 4 d/8 d, insensitive to many organics	Med.	II	Trevors et al., 1983 Blune et al., 1985
PROTOZOAN	metabolism		1.0	10 min	oxygen meter	comparable to fish opercular rhythms	not sensitive to low concentrations	Low	II	Slabbert and Morgan, 1982
BACTERIAL	enumeration		1.9	18-48 h	microbiological equipment	can provide direct information on contamination	stressed bacteria are much more difficult to isolate, numbers are affected by turbidity	Med.	III	Dutka and Kwan, 1981, 1982, 1988 Dutka et al., 1983 Dutka et al., 1986, 1988a,b
	natural metabolism		1.9	30 min to 3 h	oxygen meter		difficult to standardize	Low	II	
	coliphage test		1.9	6-24 h	microbiological equipment	provides information on viral and bacterial pathogens	turbidity decreases numbers up to 90%	Med.	II	Dutka et al., 1986, 1988a,b
	sterol levels in sediments		2.7			mammalian feces only source	levels require good preservation, only good for organic sewage not amenable to random, one-time sample testing	Low	III	Dutka et al. 1986, 1988a,b Dutka and Rao, 1988
	growth	ECHA Biocide Monitor	1.3	18-24 h	nose	cheap, portable, no training, commercially available	not sensitive to chlorinated phenolics, chlorine or chlorine-release components	High	I	Dutka and Gorrie, 1989
	CO_2 production		1.9	3-20 min	CO_2 sensing electrode		many substances interfere with CO_2 production	Med.	II	Doward and Barua, 1984
	dehydrogenase activity	Resazurin Reduction	1.3	20-30 min	spectrophotometer	low coefficient of variation, stable, reproducible, commercially available	subject to effects of modifying factors	High	I	Liu, 1986
	motility		1.0	2 h	microscope		need training, subjective, bacterial culture required, is very sensitive to stress	Med.	II	Dutka and Kwan, 1982 Coleman and Qureshi, 1985
	beta-galactosidase activity	Toxichrome	1.3	60-90 min	spectrophotometer	results similar to Microtox, available commercially	not sensitive to all compounds	High	I	Reinhartz et al., 1987
	SOS-DNA repair	SOS-Chromotest	2.4	2 h but requires overnight bacterial pre-incubation	ELISA photometer or spectrophotometer	more sensitive than Microtox commercially available	does not work for protein inhibitors	High	III	Xu et al., 1987 Vigerstad et al., 1987 Quillardet et al., 1982, 1985
	ATP changes	ATP-TOX	2.4	5-8 h	Turner luminometer	commercially available	many steps and manipulations, different species have individual sensitivity spectrum	High	II	Xu and Dutka, 1987a,b
	fluorescence	Microtox	1.3	5-30 min	Microtox reader	commercially available	not sensitive to all compounds	High	I	Bulich et al., 1981
	luminescence	Tchan	1.3	1-24 h	photobioluminination	sensitive to photosynthetic inhibitors	not as sensitive as Microtox or fish	High	II	McFeters et al., 1983

1. Environmental Relevance Factors

High: directly comparable to laboratory macrobioassay tests.

Medium: comparable to other microassays (indirectly comparable to macrobioassay tests).

Low: shows comparability with some measure of toxicant impact (i.e., fish opercular rhythms).

2. Category:

I - very useful as field microassay for screening purposes.

II - useful as field microassay, but use is restricted to certain chemicals or indirectly comparable.

III - useful for screening, but not applicable to field, or of unknown relevance.

IV - not useful as field microbioassay.

Table 4. Summary of categorization[a] of microassays and their average ranking (from Table 3).

Environmental Relevance	High	Medium	Low
Category I	Bacterial fluorescence (1.3) Bacterial inhibition (1.3) Bacterial enzyme activity (1.3)		Bacterial enumeration (1.9)
Category II	Bacterial ATP (2.4)	Algal metabolism (2.4) Bacterial nitrification (1.6) Bacterial respiration (1.3-1.9)	Protozoan respiration (1.0) Bacterial metabolism - natural (1.0 - 1.9)
Category III	SOS - chromotest (2.4)	Bacterial growth (1.0 - 2.4) Bacterial motility (2.4)	
Category IV	Botanical tests[b] Invertebrate tests[b] Vertebrate tests[b] Algal growth[b] Protozoan growth[b]	Protozoan growth inhibition (3.3) Coliphage tests[b]	Sediment sterol levels (2.7)[b]

[a] **Environmental Relevance Factor:**

High: - directly comparable to laboratory macrobioassay tests.

Medium: - comparable to other microtests (indirectly comparable to macrobioassay tests).

Low: - shows comparability with some measure of toxicant impact (i.e., fish opercular rhythms).

Category:

I - very useful as field microassay for screening purposes.

II - useful as field microassay, but use is restricted to certain chemicals indirectly comparable or of unknown relevance.

III - useful as field microassay, but limited applicability to field and of unknown relevance.

IV - not useful as field microassay.

[b] not ranked because the assays generally require too much time to complete (usually 48 or 96 h).

II) and the SOS-Chromotest (category III) (Table 3). The correlations of these tests with accepted "legal" bioassay tests are presented. Since data on *Daphnia* and rainbow trout were not available for all microassays, all available data were presented. The chemical groupings were: a) pure organic chemicals; b) inorganic chemicals; c) municipal wastes; d) oil refinery wastewaters; e) industrial effluents/complex wastes and f) sediment extracts or landfill leachates. A summary is included at the end of the Microtox discussion.

3.1 Category I Tests

3.1.1 Microtox

Most of the available data discussing correlations of microassays with aquatic toxicity tests deal with the Microtox assay. Comparison for Microtox correlations have been separated into groups based on chemical characteristics.

1. Organic Chemicals

Comparative investigations of the effects of organics on Microtox have been documented by Shiotsuka et at. (1980), Bulich et al. (1981), Chang et al. (1981), Curtis et al. (1982), Qureshi et al. (1982), Yates and Porter (1982), Casseri et al. (1983), Ribo and Kaiser (1983), Indorato et al. (1984), Strosher (1984), Greene et al. (1985), Hermens et al. (1985), Nacci et al. (1986), Reteuna et al. (1986), Tarkpea et al. (1986), Vasseur et al. (1986), Williams et al. (1986b), Surowitz et al. (1987), Elnabarawy et al. (1988), Parrott (1988) and Walker (1988). Kaiser and Ribo (1988) compiled EC50 data on the impacts of 600 chemicals on the Microtox assay. The organic investigations have included chlorinated toluenes, benzenes and phenols, detergents, dyes, metallurgical fluids, pharmaceuticals and mycotoxins.

In general, Microtox measurements of the toxicity of organic chemicals with non-specific modes of action are measured similarly by *Daphnia*, fathead minnow lethality, and sea urchin tests (Nacci et al., 1986),

Daphnia and *Chironomus tetans* lethality (Giesy et al., 1988) and in saline waters (marine) algal, crustacean and fish lethality (Vasseur et al., 1984a).

Daphnia are more sensitive than Microtox for simple organic compounds, such as ammonia (1.9 to 28x more sensitive), cyanide (2.2 to 28), hexachloroethane (5.9), pentadione (7.8) and sodium lauryl sulphate (2 to 3). *Daphnia* showed no discernable difference in sensitivity to propanolol, PCP, toluene and some mono/di-chlorinated benzenes, phenols and ethanols (Table 5). Microtox was more sensitive than *Daphnia* to chloroform, multi-chlorinated benzenes, phenols and ethanols, substituted pentadiones and styrene.

For single organic chemicals, rainbow trout were more sensitive than Microtox to total ammonia (58x), cyanide (89), ethylacetate (23), ethylpropionate (15), propanolol (6), chloroform (14), acetone (4), benzene (4), phenol (2) and styrene (2), but showed no difference to dichloroethane (0.8) (Table 5). Fathead minnows were more sensitive than Microtox to cyanide (14), chloroethanol (10), hexachlorethane (6.4) benzene (3), pentadione (2.6) and acetone (2), but less sensitive than Microtox to multichlorinated phenols and ethanol, substituted pentadiones and sodium lauryl sulphate.

From review articles, there are claims that Microtox is more sensitive or as sensitive as "fish" (see Section 1.5) to formaldehyde (Bulich et al., 1981; Chang et al., 1981), ethanol (Chang et al., 1981), alcohols and ketones (Curtis et al., 1982), diesel-related hydrocarbons (Baker and Griffiths, 1984; Stosher, 1984; Bauer and Capone, 1985), metal-working fluid preservatives (Mallak and Brunker, 1984), and antibiotics (De Zwart and Sloof, 1983). In most cases, the data is not substantial or poorly cited (c.f. Section 1.5). From similar reviews, Microtox has been described as less sensitive than other assays to herbicides (diuron, monouron, simazine; McFeters et al., 1983; Beckmann et al., 1984), pesticides (malathion, lindane; Jonas et al., 1984; Hermens et al., 1985), nitriloacetic acid (Surowitz et al., 1987), aniline dyes (De Zwart and Sloof 1983), and a growth-stimulating factor (Baker and Griffiths, 1984).

Table 5. Relative toxicity of pure organic chemicals to Microtox, Daphnia, rainbow trout and fathead minnows. Values in brackets represent the sensitivity of the assay relative to Microtox. Values greater than one signify that Microtox was less sensitive; values less than one signify that Microtox was more sensitive.

Single Chemical	Microtox EC50 (mg/L) 5 min	15 min	30 min	Daphnia magna 48-h LC50 (mg/L)	Rainbow trout 96-h LC50 (mg/L)	Fathead minnow 96-h LC50 (mg/L)	Reference(s)
acetone	22270	28940		15800 (1.8)	7400[f] (3.9)	15000[f] (1.9)	De Zwart and Sloof, 1983; Sloof et al., 1983
ethylacetate	5160	5870		590 (9.9)	260[f] (22.6)	270[f] (21.7)	De Swart and Sloof, 1983; Sloof et al., 1983
allylamine	19.9	16.3		39 (0.4)	15[f] (1.1)	2.1[f] (0.6)	De Zwart and Sloof, 1983; Sloof et al., 1983
aniline	425	488		0.64 (>750)	43[f] (11.3)	65[f] (7.5)	De Zwart and Sloof, 1983; Sloof et al., 1983
ammonia (total)	3607			129 (28)	62 (58)		Qureshi et al., 1982
ammonia (unionized)	1.5			0.8 (1.9)	1.4 (1.0)		Qureshi et al., 1982
benzene	214	238		400 (0.6)	56[f] (4.3)	84[f] (2.8)	De Zwart and Sloof, 1983; Sloof et al., 1983
Chlorobenzenes							
mono	1.08	0.99	1.00	0.12 (0.1)			Ribo and Kaiser, 1983[a]
1,2	1.73	1.67	1.56	1.79 (1.1)			
1,3	1.68	1.55	1.46	0.72 (0.2)			
1,4	1.53	1.48	1.44	1.13 (0.5)			
1,2,4	1.89	1.69	1.66	0.56 (0.3)			
1,2,3,5	1.82	1.79	1.94	1.34 (0.35)			
1,2,4,5	1.33	1.52	1.68	-0.39 (0.019)			
benzonitrile	19				32 (0.6)	64 (0.30)	Lebsack et al., 1981
chloroform	435			758 (0.6)	32 (13.6)		Qureshi et al., 1982
catechol	32				8.9 (3.6)	3.5 (9.1)	Lebsack et al., 1981
o-cresol	20.7	15.4		9.5 (1.6)	13[f] (1.2)	34[f] (0.45)	De Zwart and Sloof, 1983; Sloof et al., 1983
	32				8.4 (3.8)	18 (1.8)	Lebsack et al., 1981
m-cresol	8.2				8.9 (0.9)	56 (0.15)	Lebsack et al., 1981
p-cresol	1.3				8.6 (0.2)	29 (0.04)	Lebsack et al., 1981

Table 5 Cont'd. Page 2.

Single Chemical	Microtox EC50 (mg/L) 5 min	15 min	30 min	Daphnia magna 48-h LC50 (mg/L)	Rainbow trout 96-h LC50 (mg/L)	Fathead minnow 96-h LC50 (mg/L)	Reference(s)
cyanide	13.3 2.8-3.5			6.1 (2.2) 0.08-0.1 (28)	0.15 (89)	 0.1-0.2 (14)	Qureshi et al., 1982 Elnabarawy, 1986
2 chloroethanol		390.8		212 (1.8)		38.7 (10.1)	Nacci et al., 1986
2,2,2 trichloroethanol		43.9		148 (0.30)		173 (0.25)	Nacci et al., 1986
1,2 dichloroethane		158		1430 (0.11)	198 (0.80)		Qureshi et al., 1982
2 (2-Ethoxyethoxy) ethanol		10954		4010 (0.37)		26400 (0.41)	Nacci et al., 1986
hexachloroethane		8.3		1.4 (5.9)		1.3 (6.4)	Nacci et al., 1986
trichloroethylene	156	115		94 (1.2)	42[f] (2.7)	47[f] (2.5)	De Zwart and Sloof, 1983; Sloof et al., 1983
p-chlorobenzotrifluoride	1.57			12.4 (0.13)	13.5 (0.12)	12.0[e] (0.13)	Casseri et al., 1983
3,4 dichlorobenzotrifluoride	2.78	3.57		10.2 (0.35)	11.9 (0.3)	12.8[e] (0.3)	Casseri et al., 1983
n-heptanol	14.5	19.9		65 (0.3)	43[f] (0.5)	34[f] (0.6)	De Zwart and Sloof, 1983; Sloof et al., 1983
2,4 - pentadione		373		47.6 (7.8)		142 (2.6)	Nacci et al., 1986
2 methyl, 2,4 pentadione		1447.5		7060 (0.21)		8690 (0.17)	Nacci et al., 1986
2 methyl, 1-propanol		1224.6		1110.0 (1.1)		1510 (0.8)	Nacci et al., 1986
n-propanol	17700	18400		6300 (2.9)	3200[f] (5.8)	5000[f] (3.7)	De Zwart and Sloof, 1983; Sloof et al., 1983
ethylproprionate	612	811		250 (3.2)	56[f] (14.5)	70[f] (11.6)	De Zwart and Sloof, 1983; Sloof et al., 1983

Table 5 Cont'd. Page 3.

Single Chemical	Microtox EC50 (mg/L) 5 min	15 min	30 min	Daphnia magna 48-h LC50 (mg/L)	Rainbow trout 96-h LC50 (mg/L)	Fathead minnow 96-h LC50 (mg/L)	Reference(s)
phenol	22.0			32.0 (0.69)	9.9 (2.2)		Qureshi et al., 1982
	21 - 41			10 - 23 (1.8)		23 -24 (1.0)	Elnabarawy, 1986
	22.0 - 40.2	34.0		7.0 - 88.0	5.0 - 11.6	24.0 - 67.5	Walker, 1988
	25				8.9 (2.8)	3.5 (7.1)	Lebsack et al., 1981
Chlorophenols							
2	0.54	0.51	0.58	<0.77 (1.8)			Ribo and Kaiser, 1983[a]
4	1.18	1.15	1.19	1.17 (1.0)			
2,4	1.54	1.51	1.47	<1.21 (0.6)			
2,4,5	2.22	2.21	2.19	1.72 (0.3)			
2,4,6	1.52	1.38	1.41	1.10 (0.5)			
2,3,4,6	2.09	2.20	2.26	<2.37 (1.3)			
2,3,5,6	1.92	1.96	2.02	1.97 (1.0)			
2,3,4,5,6	2.46	2.64	2.71	2.25 (0.62)			
3,5 dichlorophenol	3.0-4.5			1.7-4.0 (1.1)		8.3-9.7 (0.54)	Elnabarawy, 19886
pentachlorophenol	0.08-0.15			0.14-0.28 (0.5)		0.2-0.5 (0.7)	Elnabarawy, 1986
		1.0		0.1 (10)		0.3 (3.3)	Nacci et al., 1986
	0.94	0.76		0.48 (1.6)	0.2[f] (3.6)	0.21[f] (3.6)	De Zwart and Sloof, 1983; Sloof et al., 1983
hydroquinone	0.079				0.097 (0.8)	0.044 (1.8)	Lebsack et al., 1981
benzoquinone	0.0085				0.13 (0.07)	0.045 (0.2)	Lebsack et al., 1981
resorcinol	310				>100 (3.1)	100 (3.1)	Lebsack et al., 1981
sodium lauryl sulfate	1.6-3.2			7.3-13 (2.3)		6.2-9.6 (0.52)	Elnabarawy, 1986
salicylaldehyde	16.3	14.3		5.8 (2.5)	1.35[f] (10.6)	4.2[f] (3.4)	De Zwart and Sloof, 1983; Sloof et al., 1983
styrene	5.4			59 (0.09)	2.5 (2.2)		Qureshi et al., 1982

Table 5 Cont'd. Page 4.

Single Chemical	Microtox EC50 (mg/L) 5 min	15 min	30 min	Daphnia magna 48-h LC50 (mg/L)	Rainbow trout 96-h LC50 (mg/L)	Fathead minnow 96-h LC50 (mg/L)	Reference(s)
toluene	20-34 30-135[b]			15-23 (1.3)	8[c] (>3.8)	23-50 (<0.07)	Elnabarawy, 1986 Vasseur et al., 1984a
o-chlorotoluene	6.14	6.19			2.3 (2.7)	7.5 (0.8)	Casseri et al., 1983

a all values expressed as log (1/Concentration)(L/mmol).
b 10 min EC50.
c Oncorhynchus gorbuscha.
d unspecified fish.
e bluegill sunfish.
f 48-h LC50.

Correlation coefficients for comparison of Microtox EC50s with other measures of toxicity are very high if organics are grouped together based on structural similarities (Table 6). Correlation coefficients do not provide any information about relative sensitivity, only about how parallel the findings are. The correlation coefficient for chlorobenzenes is poorer than chlorophenols due to their lower solubility (Ribo and Kaiser, 1983) and other authors have suggested that Microtox is not good for highly lipophilic organics (e.g., PCBs; Vitkus et al., 1985). A comparison of the slopes of the regressions shows that Microtox has similar sensitivity to *Daphnia* and the bluegill, and is slightly more sensitive than the guppy, brown trout and shrimp (Ribo and Kaiser, 1983). Correlation coefficients are much lower if large numbers of structurally different organics are grouped together (Curtis et al., 1982; Indorato et al., 1984; Tarkpea et al., 1986).

Microtox shows a decreased sensitivity to organics which have poor solubility (Bulich et al., 1981; Ribo and Kaiser 1983; Hermens et al., 1985; Vitkus et al., 1985), and to chemicals which do not penetrate cell walls easily (Surowitz et al., 1987). Microtox is generally more sensitive than activated sludge (Elnabarawy et al., 1988), *Spirillum, Pseudomonas* and dissolved oxygen techniques (Dutka and Kwan, 1984). In a comparison of the toxicity of phenol to 149 different species, Walker (1988) found that Microtox was more sensitive than all other bacterial tests (Table 7).

2. Inorganics/Metals

The impacts of metals on Microtox fluorescence have been investigated by Bulich et al., (1981), Dutka and Kwan (1982, 1984), Qureshi et al., (1984), Vasseur et al., (1984a,b, 1986), Greene et al. (1985), Reteuna et al. (1986), Tarkpea et al. (1986), Thomas et al. (1986), Surowitz et al. (1987), Elnabarawy et al. (1988), Morel et al. (1988) and Parrott (1988). Intercomparisons are complicated due to the use of different exposure times, pH and assay temperatures. For some metals, the toxicity (as assessed by the Microtox assay) increases with temperature (Vasseur et al., 1984a) and a slow, progressive response is evident for most metals regardless of temperature (Dutka and Kwan, 1982; Vasseur et al., 1984a,b; Elnabarawy, 1986).

Table 6. Correlations (r^2) between Microtox and specified bioassays for simple organic chemicals.

Response	Organism	Chlorophenols[a]	p-substituted phenols[b]	Chlorobenzenes[c]	Ketones[d]	Ethanes[e]	Alcohol[f]	Organics
log p	--	0.91[g]		0.76				
EC50	Bacillus[k]	0.93						
EC50	B. subtilis[l]	0.73						
24 h LC50	Daphnia	0.87						
48 h LC50	Daphnia			0.83				
96 h LC50	Nitocra							0.75[h]
96 h LC50	Crangon septemspinosus	0.68						
24 h LC50	brown trout	0.92						
24 h LC50	bluegill	0.77						
96 h LC50	bluegill			0.71				
95 h LC50	sheepshead			0.80				
96 h LC50	fathead minnow				0.81	0.99	0.96	0.91[i]/0.72[j]
96 h LC50	golden orfe							0.71[i]
7-14 d LC50	guppy (static)	0.89		0.86				
ip LD50[m]	rainbow trout		0.93	0.74				

a 30 min EC50, n=20, Ribo and Kaiser, 1983
b 30 min EC50, n=13, Ribo and Kaiser, 1983
c 30 min EC50, n=12, Ribo and Kaiser, 1983
d Curtis et al., 1982, n=7
e Curtis et al., 1982, n=4
f Curtis et al., 1982, n=8
g Without ortho-substituted phenols
h Tarkpea et al., 1986
i Indorato et al., 1984
j Curtis et al., 1982, n=68
k Dehydrogenase activity
l Spore germination
m Intraperitoneal injection

Table 7. Comparison of phenol toxicity to Microtox with other studies (modified from Walker, 1988). The range of phenol toxicity to Microtox was 22.0 to 40.2 mg/L for 8 laboratories (mean = 29.4). Walker's (1988) comparison included 149 species and 234 tests.

Response	Test	Phenol (mg/L)
EC50	Microtox	29.4
	Tchan	880
	Activated Sludge	900
	Bacterial electrode	1209
	BOD inhibition	225
	Glucose-uptake incubation	650
	Oxygen depletion	775
	Oxygen uptake	1200
	Growth *P. putida* (20-h/48-h)	315/880
	P. fluorescens	480
	Resazurin reduction 30 min	2300
	90 min	74.3
	Spirillus (5 min)	400
	120 min	198
	Spore germination	142.3
	TTC reduction	1400
LC50	fish	2.5 to 67.5
	bivalves	94 to 100
	crustaceans	15 to 183
	worms and leeches	100 to 1280
	insects	2.0 to 220[a]

[a] without mayflies and caddisflies the range would be 16 to 2000.

In general, Microtox is not as sensitive to inorganic chemicals as *Daphnia* or the rainbow trout, but is comparable to the fathead minnow (Table 8). *Daphnia* were more sensitive than Microtox to copper (60 to 370x), chromium (100), cadmium (>60), arsenate (6.5), zinc (2.0 to 9.6), mercury (1.0 to 2.7) and cobalt (1.2). Rainbow trout were more sensitive to cadmium (400), copper (30) and zinc (22), but less sensitive to mercury (0.38) and arsenate (0.81). Fathead minnow were more sensitive to cadmium (<100), and of approximately the same sensitivity as Microtox to chromium (1.0 to 1.9), copper (0.006 to >40) and zinc (0.1 to 1.2), but less sensitive to cobalt (0.32) and mercury (0.44). In comparison with other bacterial assays, Microtox also showed a lower sensitivity to lead (Dorward and Barisas, 1984), cadmium (De Zwart and Sloof, 1983) and nickel (Dutka and Kwan, 1984) (Table 9).

3. Municipal Wastes

The use of Microtox for monitoring the toxicity of sewage treatment wastes has been reported by Qureshi et al. (1982), Rowlen et al. (1983), Dutka and Kwan (1984), Robinson (1988) and Slattery (1988). Examinations of municipal waste toxicity generally provide long tables offering comparisons of EC50 data, but have not quantified the chemical constituents of waste water to permit quantitative analysis of relative differences. Rowlen et al. (1983) found that Microtox was more sensitive than the rainbow trout assay, although Qureshi et al. (1982) did not find major differences (Table 10). Dutka and Kwan (1984) found that Microtox was more sensitive than either the *Spirillum* motility assay or measurement of activated sludge respiration (Table 10). Although the three tests were in total agreement on toxic/non-toxic designations, activated sludge measurements were more than 100x less sensitive with respect to EC50s.

Robinson (1988) found that the sensitivity of Microtox was much superior than that of activated sludge respiration, nitrification or bacterial colony growth, and the coefficient of variation was less than half. Slattery (1988) divided the toxicity associated with sewage effluents into particulate and soluble toxicity, and found that Microtox responded to the soluble toxicity, but was not very sensitive to the toxicity associated with particulates.

Table 8. **Relative toxicity of metals to to Microtox, Daphnia, rainbow trout and fathead minnows. Values in brackets represent the sensitivity of the assay relative to Microtox. Values greater than one signify that Microtox was less sensitive; values less than one signify that Microtox was more sensitive.**

Single Chemical	Microtox EC50 (mg/L) 5 min	15 min	30 min	Daphnia magna 48-h LC50 (mg/L)	Rainbow trout 96-h LC50 (mg/L)	Fathead minnow 96-h LC50 (mg/L)	Reference
Sodium arsenate (As^{+5})	35			5.4 (6.5)	43 (0.81)		Qureshi et al., 1982
Cadmium (Cd^{+2})	70 - 90	10		0.02 - 0.16 (>60)		0.01 - 0.14 (>70)	Elnabarawy, 1986
			7 - 60			55[a] (1 - 8)	Vasseur et al., 1984a
	1070	218					De Zwart and Sloof, 1983
				0.046 (>4500)	0.15[c] (>400)	2.2[c] (100)	Sloof et al., 1983
Chromium (Cr^{+6})	70 - 100	13		0.10 - 0.13 (100)		12 - 53 (1.0)	Elnabarawy, 1986
			42 - 58			31[b] (1.4 - 1.9)	Vasseur et al., 1984a
Cobalt	135 - 177	16		4.7 - 13 (1.2)		50 - 70 (0.32)	Elnabarawy, 1986
Copper	7.4			0.02 (370)	0.25 (30)		Qureshi et al., 1982
	4 - 20			0.01 - 0.06 (>67)		0.02 - 0.10 (>40)	Elnabarawy, 1986
			0.5 - 2.0			78[a] (0.006)	Vasseur et al., 1984a
Mercury	0.08			0.03 (2.7)	0.21 (0.38)		Qureshi et al., 1982
	0.03 - 0.07			0.01 - 0.06 (1.0)		0.16 - 0.66 (0.44)	Elnabarawy, 1986
	0.044 - .032						De Zwart and Sloof, 1983
				0.005 (6.4)	0.65[c] (0.5)	0.05[c] (0.64)	Sloof et al., 1983
Zinc	49			5.1 (9.6)	2.2 (22)		Qureshi et al., 1982
	2 - 14			1.0 - 1.2 (2.0)		0.5 - 1.7 (>1.2)	Elnabarawy, 1986
		1.4 - 8				66 (<0.1)	Vasseur et al., 1984a

a Fundulus heteroclitus
b Aldrichetta fosteri
c 48-h LC50

Table 9. Relative sensitivity of Microtox to metals, compared to specified bioassays[a,b].

Metal	More Sensitive Tests	Same Sensitivity	Less Sensitive Tests
Cu	*Daphnia*, FHM algae, rainbow trout	rainbow trout[a]	*Spirillum*, *Pseudomonas*, activated sludge TTC, RR, root elongation, earthworm, polytox
Cd	*Daphnia*, FHM, algae		activated sludge, TTC, RR, *Nitocra*, root elongation, earthworm, polytox
Co	*Daphnia*		FHM, activated sludge, TTC, RR polytox
Ni	Activated sludge *Pseudomonas*, *Spirillum*		
Zn	*Daphnia*, rainbow trout, FHM, activated sludge, algae	*Daphnia*[a]	*Spirillum*, *Pseudomonas*, activated sludge, TTC, RR, root elongation, earthworm, polytox
Pb	*Pseudomonas*		*Spirillum*, activated sludge, polytox
Hg		rainbow trout *Daphnia*	*Nitocra*, FHM, RR, TTC, activated sludge, polytox
As	*Nitocra*	rainbow trout	polytox, activated sludge
Cr^{+6}	*Daphnia*, FHM		activated sludge, TTC, RR, polytox

[a] names appearing more than once in a comparison were ranked differently by different authors

[b] Abbreviations used: FHM - 96-h LC50 for fathead minnow
TTC - dehydrogenase activity
RR - dehydrogenase activity

Table 10. Comparison of Microtox with other bioassays[a] used for detecting toxicity of sewage treatment plant effluents.

Toxicant	Tests Examined	Outcome	Reference
sewage effluent	Microtox, rainbow trout	Average Mtx EC50 for 1982 and 1983, 24.8% ± 8.2; Average rt EC50 88.1% ± 11.4, for other STPs, Mtx 13.1 and rt 74.0; Mtx 98.1 and rt 122.3	Rowlen et al., 1983
sewage effluent	Microtox, rainbow trout, *Daphnia*	3 effluents examined, 2 NL and one *Daphnia* (LC50=16%) Microtox (30%) and rt (43%)	Qureshi et al., 1982
sewage effluent	Microtox, *P. flucrescens*, *S. volutans*, activated sludge	Mtx, *P. flu* > *S. vol* > activ. sludge[b]	Dutka and Kwan, 1984
sewage effluent	Microtox, bacterial growth, nitrification, respiration A (30 min), respiration B (3 h)	Mtx >> Res B > nitrit > growth, Res A[b]	Robinson, 1988
sewage effluent	Microtox, oxygen consumption	Mtx good for soluble toxicity, not for toxicity associated with particulates	Slattery, 1988

[a] abbreviations used:
Mtx - Microtox
rt - rainbow trout
STP - sewage treatment plant
NL - non-lethal
P. flu - *Pseudomonus fluorescens*
S. vol - *Spirillum volutans*
Res A - Respiration A (30 min)
Res B - Respiration B (3 h)
nitrit - nitrification
activ. sludge - activated sludge respiration

[b] For long lists of comparisons not involving fish or *Daphnia*, the relative sensitivities were ranked non-parametrically for each chemical by the authors of this report, and the sums of rankings were used to assign relative sensitivity.

4. Oil Refinery Wastewaters

The use of Microtox for oil refinery wastewater has been examined by Burks et al. (1981), Lebsack et al. (1981), MacKinnon and Retallack (1982), Peake and MacLean (1982), Qureshi et al. (1982), Matthews and Kerr (1983), Delistraty (1984), Strosher (1984) and MacKinnon and Boerger (1986). In a Canadian Petroleum Association study (cited in Bulich, 1982) of waste drilling fluids (129 assays of 29 shared samples), the average coefficient of variation was 11% for 5 min Microtox assays and 13% for 15 min readings. This was about 1/3 of the variability evident in fish studies. In a similar study reported by Strosher (1984), 6 laboratories examined 48 waste drilling fluid samples and found a very high correlation between 96 h rainbow trout LC50 studies and Microtox ($r = 0.89$); in 13% of the tests, the bacteria were more responsive (Table 11). In a study of two oil refinery effluents, Microtox EC50s were much more sensitive indicators of toxicity than either *Daphnia* or rainbow trout (10 x difference) (Qureshi et al., 1982).

For most comparative studies of complex wastes, lists of LC50/EC50s or figures are produced which do not allow more than a subjective ranking of sensitivity. In a study of oil sands tailings, toxicity as determined by the Microtox assay was similar to *Daphnia magna*, but both tests were less sensitive than either the fathead minnow or rainbow trout test (MacKinnon and Retallack, 1982). In a follow-up study (MacKinnon and Boerger, 1986), Microtox showed decreased sensitivity relative to *Daphnia* and rainbow trout, but the EC20 calculated from the Microtox assay was greater or equal in sensitivity to that of rainbow trout and *Daphnia*.

In a study of oil refinery wastewater, Microtox and *Daphnia* were determined to be more sensitive than fathead minnow bioassays (Burks et al., 1981). Although there was a good correlation between *Daphnia* and the fathead minnow bioassays, *Daphnia* showed increased sensitivity when there were differences. When toxic/non-toxic designations were used, Microtox and *Daphnia* agreed 9/11 times (Burks et al., 1981).

Table 11. **Comparison of Microtox with specified bioassays[a] for sensitivity in detection of wastewater toxicity associated with the petroleum industry.**

Toxicant	Tests Examined	Outcome	Reference
waste drilling fluids	rainbow trout, Mtx, seed germination	Mtx: rt, r = 0.89 and Mtx, on the average 13% more responsive and less variable.	Strosher, 1984
oil sands tailings water	rainbow trout, *Daphnia*, Mtx, FHM	based on a Figure, rt were more sensitive than FHM (lower % effluent at LC50) then *D. magna*. Mtx showed the highest EC50s.	MacKinnon and Retallack, 1982
oil sands tailings water	rainbow trout, *Daphnia*, Mtx	based on tabulated bioassays, Mtx EC20 detected more positive responses than either *Daphnia* or rt, the Mtx EC50 was less sensitive than either test.	MacKinnon and Boerger, 1986
oil refinery wastewater	Mtx, rainbow trout, *Daphnia*	two effluents examined and Microtox was more sensitive then either rt or *Daphnia*	Qureshi et al., 1982
oil refinery wastewater	*Daphnia*, Mtx, FHM	*Daphnia* positive 4/11; Mtx 2/13; *FHM 4/11 (1981)* *Daphnia* 6/8; Mtx and FHM 2/8 (1982)	Burks et al., 1981
fossil fuel process waters	Mtx, rainbow trout, FHM	All samples were highly lethal; Mtx EC50 < FHM 7/16 times, FHM < Mtx 8/16 times, rt < Mtx 6/7 times.	Lebsack et al., 1981
oil waste water	Mtx	Mtx documented recovery as process waters were cleaned by organic extraction	Peake and MacLean, 1982

a abbreviations used: Mtx - microtox
rt - rainbow trout
FHM - fathead minnow

The Microtox assay has also been successfully used to document the recovery of water quality as wastewaters are cleaned up. Peake and MacLean (1982) attributed the high correlation between Microtox and sample clean-up to the relationship of the toxicity to volatile and extractable organics, and the relatively low contribution of inorganics to the observed toxicity. Symons and Sims (1988) found that Microtox values followed PAH degradation closely. Similar findings were reported by others (Casseri et al., 1983; Matthews and Bulich, 1986) who found Microtox to be as reliable as estimates of total organic carbon during clean-up.

5. Industrial Effluent/Complex Wastes

Investigations of the utility of Microtox for examining industrial effluents have been made by Bulich et al. (1981), Bulich (1982), Qureshi et al. (1982), Casseri et al. (1983), Neiheisel et al. (1983), Mallak and Brunker (1984), Calleja et al. (1986), Elnabarawy (1986), Tarkpea et al. (1986) and Blaise et al. (1987, 1988). In almost all studies (9/10), no chemical analyses were completed on the effluent samples. Most comparisons were conducted using relative agreement on toxic/non-toxic designations. Bulich (1982) summarized the impacts of 257 complex effluents and found that in a rank comparison, Microtox and fish assays showed 78% agreement, Microtox and *Daphnia* 63%, and *Daphnia* and fish 69% (Table 12). If rankings were based on assignment of toxic/non-toxic rankings of the effluent being lethal at 25% dilution, agreements were 85-89%, while at a level of 50% lethality, Microtox compared much better with fish (87%) than *Daphnia* (50%).

Calleja et al. (1986) found very similar results, but found a higher correlation at the 50% lethality designation, where the agreement between *Daphnia* and Microtox was >90%. For pulp and paper wastes, rainbow trout and Microtox showed 84% agreement, algal toxicity and Microtox 90% agreement and algal and fish toxicity 85% agreement (Blaise et al., 1987). Qureshi et al. (1982) found Microtox to be 4-8 x more sensitive than rainbow trout to pulp mill effluent, and 10 x more sensitive than *Daphnia*.

Table 12. Utility of Microtox for examing industrial effluents relative to other bioassays[a].

Toxicant	Tests Examined	Outcome	Reference
wastewater +16 unidentified organics	rainbow trout, Microtox, fathead minnow	range of MTX EC50s (>6.3 to 15.7%) lower than FHM (20.9 to >50%) and *Daphnia* (14.2 to 66.3%)	Neiheisel et al., 1983
257 complex effluents	fish, Microtox, *Daphnia*	Mtx: fish 89-87% agreement Mtx: *Daphnia* 85-75% agreement[b]	Bulich, 1982
complex effluents	Microtox, *Daphnia*	Mtx, *Daphnia* 77-91% agreement	Calleja et al., 1986
complex wastes	Microtox, rainbow trout, *Daphnia*	relative sensitivity varied between effluent types	Qureshi et al., 1982
complex effluents	Microtox, *Daphnia*, fathead minnow	Mtx predicted 81% of samples toxic to FHM and 62% of those toxic to *Daphnia*	Dutka and Kwan, 1981
pulp and paper wastes	Microtox, rainbow trout, *Selenastrum*	algae positive 54/55 times; rt 46/55, Mtx 43/51; rank agreement rt: Mtx > 84%, class agreement[c] 70% within 0.5 log class; Mtx 4x as sensitive as rainbow trout	Blaise et al., 1987
pulp and paper waste	Microtox, rainbow trout, *Daphnia*	two effluents examined and Microtox 4-6x more sensitive	Qureshi et al., 1982
industrial effluents	Microtox, *Daphnia*	*Daphnia* positive 22/39 times; Mtx 19/39 and in agreement 86% of the time.	Vasseur et al., 1984b
complex effluents	Microtox, *Spirillum*	Mtx > *Spirillum* but 90% agreement	Coleman and Qureshi, 1985
complex effluents	Microtox	Mtx less sensitive to CN, urea ethanol, NH_3	Bulich et al., 1981
complex effluents	Microtox, *Nitrocra*	Mtx more sensitive than *Nitocra*	Tarkpea et al., 1986

a abbreviations used: Mtx - Microtox
rt - rainbow trout
FHM - fathead minnow

b agreement based on toxic/non-toxic designation at 25% or 50% lethality.

c ranked as toxic (LC50 <25%), slightly toxic (LC50 25 to 100%) or non-toxic (>100%) or on class log interval (<1%; 1 to 3.2%; 3.2 to 10%; etc.).

Microtox predicted 81% of effluents found to be toxic to fathead minnows, and 62% to Daphnia (Dutka and Kwan, 1981) but does not appear to be sensitive to effluents containing high levels of cyanide, urea, ethanol or ammonia (Bulich et al., 1981). For 39 industrial effluents, Microtox showed 86% agreement with *Daphnia* EC50 (immobility) but the coefficient of variation was only 3% (relative to 30% for *Daphnia*) (Vasseur et al., 1984b). Microtox showed a greater sensitivity to phenol-based effluents, variable sensitivity to dyes and lesser sensitivity to pharmaceuticals (Qureshi et al., 1982; Vasseur et al., 1984a,b).

Microtox was found to be more sensitive than 96 h LC50s for a Swedish copecod (*Nitocra*) in testing with effluent and organics (Tarkpea et al., 1986), more sensitive than rainbow trout or fathead minnow to effluents with 16 different (unidentified) organics (Neihesel et al., 1983) and more sensitive than *Spirillum* motility to complex effluents (Coleman and Qureshi, 1985).

6. Sediment Extracts and Leachates

Microtox has been used to monitor sediment toxicity by Dutka et al. (1986, 1988a,b) and Kaiser et al. (1987, 1988), as well as Hamdy and Post (1985), Hermens et al. (1985), Kwiatkowski and Nadeau (1985), Schiewe et al. (1985), Calleja et al. (1986), Williams et al. (1986b), Symons and Sims (1988) and True and Hayward (1988) (Table 13). These studies seldom quantified contaminants present in the leachates, and compared toxicity on the basis of positive response versus negative, or toxic/non-toxic designations.

Although numerous attempts have been made to examine sediment-associated toxicity, the issue is controversial and can be complicated. Microtox is much more sensitive to organic extracts using 1% DMSO than water extracts (Dutka and Kwan, 1988), but interpretation must be made carefully. Problems exist with organic extraction techniques and the toxicity of the solvent carrier. In laboratory studies, DMSO at the 1% level was not toxic to microassays used by Dutka and Kwan (1988a,b) in their screening battery and no synergistic effects were detected between the toxicant and 1% DMSO solution. Dutka (1988) cautions that a) more rigorous extraction procedures may measure toxicants which are not biologically available, b)

Table 13. Sensitivity of Microtox for examining sediment - associated contaminants relative to specified bioassays[a].

Toxicant	Tests Examined	Outcome	Reference
Detroit River sediments	Microtox, *Daphnia*, *Chironomus tentans*	positive responses: *C. tent.* 5/30, *Daphnia* 13/30, Microtox 7/30	Giesy et al., 1988
phenol-based wastewater leachates	Microtox, *Daphnia*, fathead minnow	12/15 Mtx and 6/7 FHM negative; *Daphnia*: Mtx agreed 4/4 times but *Daphnia* 2 to 20x more sensitive. FHM : Mtx agreed 5/7 but FHM always more sensitive (<2x).	Casseri et al., 1983
Sediment extracts	Microtox, *Daphnia*, ECHA	*Daphnia* positive in 63/94, ECHA 27/94, Mtx 0/94	Dutka and Gorrie, 1989
Hazardous waste leachate	Microtox, *Daphnia*	Pesticide and electroplating sludge slightly lethal to both but, EC50 of *Daphnia* 42 to 3000X lower	Calleja et al., 1986
Lake Ontario sediments	Various	Mtx negative at all 51 stations for water samples and for 36/51 sediment samples	Dutka et al., 1986
Lake Ontario sediments	Microtox, ATP-TOX, SOS-Chromotest, *Daphnia*	Daphnia positive in 4/19 water extracts but all others negative. With organic extracts (1% DMSO), Mtx positive 18/19, ATP TOX 16/19, SOS - 0/19	Dutka and Kwan, 1988
Great Lakes sediments	Microtox, *Spirillum volutans*, activ. sludge (TTC)	Mtx > *Spir.* >>> activ. sludge[b]	Dutka et al., 1983
PEI sediments	Microtox, SOS-Chromotest	SOS more sensitive than Mtx	Xu et al., 1987
Petroleum soil wastes and aqueous soil extracts	Microtox, activated sludge	Mtx more sensitive than activ. sludge[b]	Symons and Sims, 1988
Sediment extracts	Microtox, amphipod mortality oyster deformity	Mtx negative 29/50; amphipod 17/50; oyster 16/50 Mtx:amphipod r=0.48; Mtx:oyster r=0.62; oyster:amphipod r=0.86	Williams et al., 1986b
Puget Sound sediment	Microtox	good for ranking sediments	Schiewe et al., 1985
Synfuel by-product waters	Microtox	good for examining relative toxicity	Delistraty, 1984

a abbreviations used: Mtx - Microtox
C. tent. - *Chironomus tentans*
Spir. - *Spirillum*
activ. sludge - Activated sludge
SOS - SOS-Chromotest

b - For comparisons not involving fish or *Daphnia*, the relative sensitivities were ranked non-parametrically for each chemical by the authors, and the sums of the rankings were used to assign relative sensitivity.

the solvent may potentiate the response and c) increasing the complexity of the test also increases the expense and time commitment to procedures which are only meant for monitoring and prioritization. Several authors only use pore water extract as an estimate of biologically-available toxicants (e.g., Giesy et al., 1988).

True and Hayward (1988) found that Microtox has the potential to estimate both water soluble and solvent soluble compounds in marine sediments, if the extractions are done differently. They recommend separating toxicity into the portion associated with interstitial water and toxicity associated with the solvent extractable portion of the sediment. The solvent extractable portion can be determined by methylene chloride extraction, with subsequent solvent transfer by evaporation and redilution in ethanol. Schiewe et al. (1985) also recommended the transfer of methanol-dichloromethane-extracted toxicants into ethanol since ethanol is 10 times less toxic to Microtox than methanol-dichloromethane. This extraction is effective for neutral, non-ionic compounds such as aromatic and chlorinated hydrocarbons, and naphthalene (Schiewe et al., 1985; Anon, 1986). Some authors have recommended saline-extraction of sediments, but the saline may stimulate *P. phosphoreum* metabolism (Kaiser et al., 1988). Comparisons with volatile halocarbons, organochlorines, PCBs and PAHs have been of limited success although the extracts are good for chlorinated phenols (Ribo et al., 1985). This may be due to the very low acute toxicity demonstrated for highly lipophilic compounds (Ribo and Kaiser, 1983; Hermens et al., 1985; Vitkus et al., 1985).

Ribo et al. (1985) recommended the use of rotary evaporation and fractionation to examine samples. The procedure is not effective for metals, organo-metals, electrolytes, pH and surfactants and can only be used to identify relative differences. Results were comparable to levels of volatile halocarbons which have been considered a general indicator of contaminant levels (Ribo et al., 1985).

In interassay comparisons involving sediment extracts, Microtox was 875x less sensitive (based on differences in EC/LC50s; range 42-3000) than *Daphnia* to leachates involving electroplating sludge (Fe, Cd, Cr, Ni, Cu,; pH 4.4-9.1) and pesticide waste (bendiocarb, propham, azinphos methyl, chloropropham, dimethoate,

dichlorvos) (Calleja et al., 1986). Microtox was also reported to be less sensitive to DDT (Calleja et al., 1986). In a large scale comparison of Superfund site toxicity, Microtox did not show a positive response 65% of the time to toxicity which was detected by *Daphnia* or algal tests (Greene, pers. comm.). In a comparison of 326 samples, 48% were toxic to algae, 41% to *Daphnia* and 21% to Microtox. The Microtox test did not show any toxicity response to samples of electroplating waste (Greene, pers. comm.). Of the 326 samples, Microtox did identify 4 samples as being toxic which were missed by *Daphnia* and the algal tests.

In a study of water extracts of sediments, Microtox failed to document any positive responses, although *Daphnia* was positive 4/19 times (Dutka and Kwan, 1988). Microtox did not show any positive responses to water-extracts of lake sediments, although *Daphnia* showed a response to 63 of 94 samples (Dutka and Gorrie, 1989). The ECHA dipstick was positive in 27 samples, *Spirillum* in 1, and Microtox was negative in all. Only 59% of ECHA positive responses were also positive in *Daphnia* bioassays and Dutka and Gorrie (1989) again emphasize that it is unwise to use a single toxicant screening procedure. Similar conclusions were arrived at by Williams et al. (1986b) who examined the effects of saline sediment extracts on Microtox, amphipod mortality and oyster abnormalities. Although Microtox showed the greatest sensitivity (positive responses to 63% of samples) compared to amphipods (35%) or oysters (41%), Microtox was poorly correlated with either test (r=0.48, 0.62, respectively). The variability was attributed to differences in exposure routes, heterogenous contaminant distribution and individual sensitivity patterns (Williams et al. 1986b). These differences are a persistent problem encountered in comparisons of different bioassay tests.

7. Microtox Summary

Microtox has a lower coefficient of variation than many other assays because of highly formalized, standardized reagents which are less susceptible to variation (McFeters et al., 1983). It is also possible that lyophilized bacteria show an increased sensitivity to some toxicants because they can cross cell walls

easily and this may account for the increase in sensitivity of Microtox to multi-chlorinated organics. The variability of Microtox is much higher with metals than with organics (Reteuna et al., 1986) (Table 14), and in general, the coefficient of variation decreases as toxicity increases (Greene et al., 1985).
Several disadvantages are still evident and are described below.

1) **Variability with time**

The variability of readings over time depends on the characteristics of the chemical. For sediment-associated contaminants, 5 min readings are generally the same as 15 min (Williams et al., 1986b) but effluents with a high inorganic content, such as electroplating wastes, show an increased sensitivity with time (Calleja et al., 1986). For other tests, the 15 or 30 min EC50 has been reported as most sensitive (Ribo and Kaiser, 1983). The most dramatic time-associated changes have been reported for metals (Cd, Se, Zn, Ni; Qureshi et al., 1983) although changes over time have been reported for a few organics such as phenol (Qureshi et al., 1983) and mycotoxins (Yates and Porter, 1982).

2) **pH adjustment**

There are problems associated with the requirements of the Microtox test for pH adjustment. Although sample pH can vary dramatically, the Microtox reaction is most sensitive and stable at pH 6.7 (Dutka and Kwan, 1982). Problems of pH adjustment are overlooked by some researchers because of the faster processing time offered by Microtox (Dutka and Kwan 1982). Others recommended that tests on complex effluents be conducted at their environmental pH to preserve sample stability (Qureshi et al., 1984; Coleman and Qureshi, 1985). Adjustment of pH can lead to metal precipitation (Qureshi et al., 1983) and alteration in metal toxicity. Although addition of a buffer corrects the problem for some metals, some buffers are toxic and most buffers result in a decrease in luminescence over time (Qureshi et al., 1983).

Table 14. The variability of Microtox assays as reported for various toxicants.

Toxicants	Coefficients of Variation (%)	Reference
Malathion	1.8	Dutka et al., 1986
Phenol	2.4	Dutka et al., 1986
Sewage	3-5	Robinson, 1988
Effluents	3	Vasseur et al., 1984b
Drilling Wastes	11-13	Bulich, 1982
Effluents	18.2	Bulich et al., 1981
Sodium lauryl sulphate	15-28	Bulich, 1982
Effluents	34	McFeters et al., 1983
Metals	30-35	Thomas et al., 1986
Metals	59 (20 to 113%)	Greene et al., 1985

3) Coloured or turbid samples

Colour correction and turbidity were problems during earlier studies (Bulich et al., 1981; Qureshi et al., 1983) but correction methods are now available (Bulich, 1986).

4) Salinity

P. phosphoreum is a marine bacterium and decreases in the NaCl content of samples to levels below 1% can disrupt more than 70% of the fluorescence (Ribo and Kaiser, 1987). The assay is also affected by salinity deviation (Krebs, 1983) but may re-equilibrate (Bulich, pers. comm. in Williams et al., 1986b).

5) Field Operations

Condensation interferes with machine operation under very humid conditions and precautions are required against vibration interference (Kaiser et al., 1988; Kaiser, pers. comm.). The toxicity of samples can be masked, at least partly, by the presence of compounds stimulating bacterial metabolism. A low or negative reading does not necessarily indicate the absence of toxic substances (Kaiser et al., 1988).

Variability in the Microtox assay has been attributed to duration of the assay, use of different formulations, pH, solubility, toxicant measurements and data analysis (Greene et al., 1985). Microtox was not included in Dutka's (1988) battery of toxicity tests because it showed a low independence relative to other tests and was not highly correlated with any one other test in the battery. There are also problems associated with the age of the bacterial reagent and end point precision. Aging of the reagent to 4 h increases the sensitivity to metals, and decreases Microtox sensitivity towards organic compounds (Qureshi et al., 1983). Seasonal changes also affect the field operation of the Microtox assay. Decaying leaf litter releases

significant amounts of lignin-derived products, such as phenolics and tannins, which exert strong, toxic effects on *P. phosphoreum* (Ribo and Kaiser, 1983; Kaiser et al., 1988).

In spite of these limitations, the Microtox assay, may be the best choice at present for field monitoring of industrial effluents. It is the most sensitive bacterial test to most organics and shows greater sensitivity to effluents than pure compounds (Tarkpea et al., 1986). Microtox is more sensitive to commercial formulations of herbicides and pesticides than pure chemical forms (Thomas et al., 1986) and shows decreasing variability in effluents (Table 14) as toxicity increases (Greene et al., 1985). Microtox is a convenient method for monitoring waste treatment (Casseri et al., 1983; Matthews and Bulich, 1986), routine monitoring of plant effluents (Symons and Sims, 1988), and identification of relative differences in spatial or temporal distribution of contaminants (Blaise et al., 1987; Kaiser et al., 1988; Kaiser and Ribo, 1988; Morel et al., 1988). The increased sensitivity to effluents may be related to pH, NH_3 or phenols in many cases (Bitton, 1983).

The U.S. EPA has recommended Microtox for testing treated effluents, for predicting land treatability of organic wastes, and for bioassessment of waste disposal sites. The American Society for Testing and Materials (ASTM) has recommended Microtox for biological testing of wastewater and leachates (Elnabarawy, 1986). In general, concentrations of waterborne toxicants away from industrial areas are too low to initiate inhibition of the fluorescence. To elevate waterborne toxicant concentrations, Dutka et al. (1986, 1988a,b) suggest flash evaporation of water samples at 45°C to achieve a ten-fold concentration of the toxicants. However, Ribo et al. (1985) found that evaporation of waterborne samples in a rotary-evaporator at 40°C decreased the toxicity of samples containing volatile phenols, at ug/L concentrations. In those cases, the distillate showed increased toxicity, and Kaiser's group recommends the simultaneous testing of the distillate when concentrating waterborne toxicants (Ribo and Kaiser, 1987).

Rather than concentrating the waterborne samples so that an EC50 can be calculated, Kaiser recommends that results be reported as gamma values (Ribo et al., 1985; Kaiser et al., 1988; Kaiser and Ribo, 1988). A disadvantage of this approach is that Microtox will commonly yield negative gamma values, which is

interpreted as low (or no) toxicity in combination with nutrient stimulation by the sample. The gamma value reflects the bacterial response to a combination of stimulating and inhibiting factors present in each sample.

Recommendations for the evaluation and refinement of Microtox include (modified from Qureshi et al., 1984):

a) metals should be assayed at their observed pH

b) freshly reconstituted bacterial should be used to eliminate false positives, and

c) 15 min time periods should be used, and

d) metal mixture studies, such as those by Hermens et al. (1985) and Parrott (1988) should be interpreted with caution.

3.1.2 Toxichrome Test

This test has been in the evaluation process for the past three years at several major laboratories, including Environment Canada in Quebec (Dr. C. Blaise's laboratory), U.S. EPA at Corvallis (Dr. J. Greene's laboratory) and the Canadian National Water Research Institute in Burlington (Dr. B. Dutka's laboratory). Published data indicate that the results for the Toxichrome test are similar to Microtox for organophosphates, carbamates, pyrethroids, chlorcarbons and mycotoxins (Reinhartz et al., 1987), all substances which Microtox is thought to have a low sensitivity to. Unpublished data show that in a comparison of 120 samples taken from Superfund sites, 65% of samples were designated as toxic by the *Daphnia* or algal bioassays, but only 2 samples were positive in the Toxichrome test. These samples were those with the highest toxicity as designated by the algal bioassay and the sample which the daphnid bioassay designated as second most toxic (Greene, pers. comm.). This suggests that the Toxichrome test has some of the same insensitivities as the Microtox test, sometimes requiring either high contaminant levels or pre-concentration of the sample to obtain positive results.

3.1.3 Dehydrogenase enzymes

In general, heavy metals (Ag, Hg, Cr) are more toxic to dehydrogenase enzymes than organics (pulp mill effluent, phenol, formaldehyde) (Bitton, 1983). Measurement of dehydrogenase activity can be affected by several variables including oxygen conditions, sediment age, extraction procedure, incubation time and diluent type as well as sediment volume (Burton and Lanza, 1985). Dehydrogenase techniques do not correlate well with macrobioassays and are generally less sensitive than Microtox (Table 9).

3.1.4 ECHA Biocide Monitor

The test shows a lot of promise for field work and can also be used for assessing sediment contaminants by submergence in the sediments for approximately 5 min. In the waterborne test, various submersion times have been tested and exposure for 60 min is superior to 10 or 1 min (Dutka and Gorrie, 1989). The ECHA dipstick showed a positive response at 27/94 sites, while *Daphnia* responded at 63/94 sites. There was agreement on toxicity at 16/27 sites that the ECHA dipstick designated as toxic (Dutka and Gorrie, 1989).

Some false negatives have been identified and the test does not seem to be sensitive to chlorinated phenolics, chlorine or chlorine-release components (Dutka and Gorrie, 1989). However, preparation of dipsticks with alternative, more sensitive species may eliminate some of these problems. For a sampling field trip of several days duration, sampling sites could easily be identified overnight. Estimated cost of the dipsticks is less than $1.00 per test, which may make it ideal for identification of "hot spots" of either waterborne or sediment contamination. However, it is too early to determine the practicality of this test until it is further developed and field tested.

3.2 Category II Tests

3.2.1 ATP-TOX

The assay has been described in detail by Xu and Dutka (1987 a,b), and the test reagents are commercially available. However, testing requires excessive sample manipulations to be really useful as a field technique and technicians require practice to obtain reproducible results (Greene, pers. comm.). Furthermore, in addition to interfering with bacterial growth and/or survival, toxicants also interfere with luciferase activity during the ATP determinations, and therefore the observed light output reduction associated with a positive result is a sum of the toxicant impacts on growth, survival and luciferase inhibition of the assay. With *E. coli*, the luciferase inhibition is believed to account for only a small contribution for most toxicants (Xu and Dutka, 1987b). Combining the results of ATP-TOX assays using *E. coli* and *P. fluorescens* brings the sensitivity of the assay up to the level of Microtox, but the Microtox assay is still faster (Xu and Dutka, 1987b). Results of ATP-TOX tests showing greater than 20% inhibition have correlated well with *Daphnia* mortality (Dutka et al., 1988b).

3.2.2 Tchan

The coefficient of variation for the Tchan microassay is relatively high compared to other assays (42%; McFeters et al., 1983). Although the test is generally not as sensitive as Microtox, advantages include an increased sensitivity to photosynthetic-inhibiting herbicides (simazine, diuron, monouron). The test is not as sensitive as fish bioassays or Microtox, except to herbicides and ethanol (McFeters et al., 1983).

3.3 Category III Tests

3.3.1 SOS-Chromotest

The SOS-Chromotest is a test for genotoxicity. Although the SOS-Chromotest assay only requires a 2 h incubation period, the test requires that bacteria be grown in culture overnight prior to the assay, and results are usually read in a photometer, although identification of toxic samples can be subjectively estimated by visual comparisons. Although the assay is available commercially from the same company as the Toxichrome test (Orgenics Ltd., Israel), the increased complexity limits its usefulness as a field microassay at present. Results are comparable to those obtained with the Ames mutagenicity test, and 90% of 180 pure chemicals tested so far have shown positive results in both the Ames and SOS-Chromotest (Vigerstad et al., 1987). Advantages of the SOS-Chromotest include the short test time span and its ideal application to situations where cell division is not possible. The results are not obscured by nutrient or microbial contamination (Quillardet et al., 1985). The SOS-Chromotest responds at lower levels of contaminants than those affecting Microtox luminescence (Dutka et al., 1986) or algal growth (Harwood et al., 1988).

The SOS-Chromotest, as it is now designed, cannot test an effluent or environmental sample at more than 4.76% of the original sample strength. Organics must be dissolved in DMSO or ethanol, and the regression analysis associated with the test commonly defines an EC50 >25% of sample strength, but the relationship cannot be further investigated (Greene, pers. comm.). Work on adapting the technique for relative toxicity analysis is currently under way. Other disadvantages include the fact that the test is subject to batch variability (Dutka et al., 1988a,b) and that the test requires overnight pre-incubation and sample preparation. As with the Ames test, this test is not sensitive to the mutagenic activity of Cr^{+3}, mycoestrogens, or oil dispersants (Vigerstad et al., 1987). This assay has shown conflicting results with the Ames test for several compounds, notably aflatoxin, benzopyrene, sodium azide and ethylene dichloride (Quillardet et al., 1985).

4.0 CONCLUSIONS

The purpose of this report was to summarize the data describing correlations between accepted "legal" bioassays (rainbow trout, *Daphnia*) and selected aquatic toxicity microscale tests.

1. The selection process reviewed more than 50 microscale tests which utilized algal, protozoan or bacterial species (summarized in Appendix A). The tests were categorized based on criteria describing portability, time, ease of use, cost and complexity. The tests selected for the highest applicability to the field were the Microtox, Toxichrome, Resazurin reduction, ECHA biocide monitor, ATP-TOX and SOS-Chromotest, all of which are available commercially.

2. As expected, most of the data available for comparisons related to the Microtox test, and correlations were separated based on chemical groupings.

   a) Organics: Microtox appears to be as sensitive as macrobioassay tests to most pure organic compounds, and several correlations were described. Microtox was generally more sensitive to more complex compounds, such as multi-chlorinated benzenes, phenols and ethanols. Microtox was not sensitive to cyanide, chloroform or phenol.

   b) Inorganics: Microtox is not as sensitive to inorganics as *Daphnia*. Microtox was also less sensitive than rainbow trout and fathead minnow, except to mercury, arsenic and cobalt.

   c) Complex Wastes: Microtox has been used with favourable results for monitoring municipal wastes, and has been very useful for monitoring relative changes in toxicity associated with petroleum wastes. In general, as the complexity and toxicity of industrial effluents increased, the correlations and sensitivity of Microtox also increased, and the variability decreased. It appears that Microtox could be used for screening of the relative toxicity of very high

toxicity, complex effluents.

d) Microtox was less sensitive than "legal" tests to insecticides, herbicides, inorganic compounds, pharmaceutical and textile effluents, and highly lipophilic contaminants. In many situations, Microtox requires sample pre-concentration or solvent extraction.

e) Microtox does have some disadvantages, including changes in sensitivity with time, pH, salinity and turbidity.

f) Recommendations for Microtox use are that:

- samples be assayed at their observation pH
- freshly reconstituted bacteria be used to eliminate false positives, and
- 15 min time periods be used, and
- studies of mixtures must be interpreted with caution.

3. Not as much information was available for the other microscale tests. The sensitivity of Toxichrome appears to be similar to Microtox, with the advantages that salinity, pH adjustment and turbidity effects are probably lower or non-existent. With respect to other category I tests, dehydrogenase activity responds well to inorganics, but has not been widely compared with macrobioassays. The new ECHA dipsticks appear to be attractive, but requires further testing and will be subject to varying sensitivity depending on the species of bacteria used. ATP-TOX is complicated, requires training and practice to run consistently, is slower than the Microtox and is not as easily adapted for field use. The SOS-Chromotest is sensitive, but needs to be adapted for relative toxicity work.

4. *Daphnia magna* usually responds to waterborne toxicant levels below those affecting Microtox, algal ATP, ATP-TOX, *Spirillum* and the SOS-Chromotest (Dutka and Kwan, 1988; Greene, pers.

comm.). It is clear that without sample pre-concentration or solvent-extraction, in most cases the microassay tests are not as sensitive as *Daphnia* (48 h test) or algal (96 h test) bioassays. In a comprehensive field study requiring the analysis of large numbers of samples, the algal and daphnid tests may also be cheaper than microassays. Tests such as Microtox and Toxichrome require constant technical supervision, whereas appropriate mobile laboratory facilities for algal or daphnid bioassays would only require personnel for short periods of time, and more than one sample could be analyzed simultaneously.

5. For field use of microassays, the best approach would involve selective use from a battery of several tests, with the final test selection dependent upon an *a priori* knowledge of the gross characteristics of the contamination involved (Table 15). Conclusions must revolve around the ability of microassays to detect relative differences in the toxic activity of samples. All tests should use algal, *Daphnia* or rainbow trout bioassays to confirm findings.

Table 15. Final summary of the suitability of selected microassays for contaminant testing.

Contaminant Type	Recommended Microassay	Comments
Relatively pure organic contaminants	Microtox	- as complexity of the waste and chemical increases, the sensitivity increases and variability decreases.
		- not as sensitive as legal bioassays for simple organics, cyanide, ammonia, urea, herbicides, pesticides or highly lipophilic compounds
		- some evidence that Microtox is more sensitive to commercial formulations than pure compounds
Inorganic contaminants or effluents	Microtox	- much less sensitive than Daphnia to copper, chromium, arsenate, zinc, mercury and cobalt
	Dehydrogenase enzymes	- appear to be sensitive but correlations with legal bioassays or Microtox not widely available
Municipal wastes	Microtox	- appears to be good for monitoring relative differences and may be more sensitive than rainbow trout
Oil refinery wastewaters	Microtox	- high correlation with rainbow trout but is less variable
		- good for monitoring relative changes
Complex wastes	Microtox	- appears to be good for pulp and paper wastes to monitor relative differences
		- not sensitive to wastes with high levels of urea, cyanide, ethanol or ammonia
		- less sensitive than "legal" bioassays to waste with high inorganic contact (chromeplating, electroplating, dyes), pharmaceuticals, pesticide, surfactants
Sediment extracts and leachates	Microtox	- results will vary with extraction technique
		- good for phenolic-based contaminants, less sensitive for other groups
		- only good for relative differences and gives many false negatives
	ECHA dipstick	- more sensitive than Microtox to sediment but not widely studied
		- may not be sensitive to chlorinated phenolics
	SOS-Chromotest	- not easily applicable to field work or relative toxicity testing
		- is a very sensitive, rapid genotoxicity test

Not enough information is available for Toxichrome, but it appears to be similar to Microtox.

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APPENDIX A

SURVEY OF MICROASSAYS

LISTING OF MICROASSAY TESTS/MACROASSAY TESTS DISCUSSED IN INTERCOMPARISONS

Brief outlines of tests which are discussed in the intercomparisons are included here to insure that the reader has some familiarity with the assays. This listing is not intended to be complete. The disadvantages or limitations are described for tests which are not considered further in the report.

1 Algal Tests

1.1 Algal Growth

1. Algal microplate technique: cell counts of any species, including *Ankistrodesmus falcatus* (Wong et al., 1983) or *Selenastrum capricornutum* (Blaise, 1986). Measurements are comparable to the results found with the ATP technique (Blaise, 1986; Blaise et al., 1986), but the test has been found to be insensitive to volatile organics in round robin inter-laboratory comparisons (Thellen et al., 1989).

2. Algal density: algal bioassays using *Selenastrum capricornutum, Scenedesmus pannonicus* or *Chlorella pyrenoidosa* monitor the impacts of 96 h exposure to toxicants on algal growth (Sloof et al., 1983). Estimates can be done with a particle counter or a haemocytometer after 48 to 192 h. Attempts to estimate density effects prior to 96 h have not been very successful (Greene, pers. comm.).

3. Algal C^{14} incorporation: a 24 h test developed by Ross (Illinois Natural History Museum, Champaign, IL) which measures C^{14} incorporation by *Selenastrum capricornutum*. The test gives results which are comparable to the Microtox assay (Burnett, pers. comm.) but is subject to the restrictions involved with using radionuclides, and is restricted to laboratory use for obvious reasons.

1.2 Electron Transport System (ETS)

Electron Transport System: also referred to as dehydrogenase activity, is monitored by the use of a redox dye (INT) in *Ankistrodesmus braunii*, *Scenedesmus quadricauda* or *Selenastrum capricornutum* (Trevors et al., 1983) similar to the techniques described in detail below for bacterial species.

1.3 Algal ATP Production

Algal ATP production: measured as an estimate of the inhibition of growth by toxicants (Blaise et al., 1984; Blaise et al., 1985). The ATP content of stressed cells are measured after 4 h, 4 d or 8 d (Blaise, 1986; Blaise et al., 1986) in terms of relative light output and compared to non-stressed controls (Dutka et al., 1986) to give an indication of acutely toxic levels required to affect growth (Dutka, 1988). The 4 h estimates are only good for flagrant toxicity (Blaise et al., 1984).

2. Vascular Plant Tests

2.1 Seed Germination Tests

Seed germination tests: correlation with fish toxicity and Microtox ($r < 0.3$) in tests with drilling wastes. The test appears to be more sensitive to inorganic wastes, and most of the toxic components of the drilling wastes were organic (Strosher, 1984).

2.2 Soybean Assay for Mutagenicity

Soybean assay for mutagenicity: seeds soaked in sludge for 24 to 48 h and seedlings scored for leaf spots (Klekowski and Levin, 1979).

3. Protozoan Tests

3.1 Respiratory Responses

Respiratory responses: measured in *Tetrahymena pyriformis* (Slabbert and Morgan, 1982) as a quick indicator of toxicity. The measurement is completed within 10 min, and gives results which are comparable to the impacts of toxicants on fish opercular rhythms. Results recorded with the protozoan were within the same range reported for changes in fish opercular behaviour (Slabbert and Morgan, 1982) or bacterial growth inhibition assays (Slabbert and Grabow, 1986). Results were compared to fish opercular rhythms after exposure to metals, cyanide, parathion and pentachlorophenol (PCP), but the responses were not the same for ammonia or phenol. Several problems are evident with this technique, including its insensitivity to low concentrations of most toxicants.

3.2 Growth Inhibition

1. Growth inhibition: protozoa are allowed to colonize artificial substrates in the field for 14 to 28 d and are then tested for growth effects in laboratory exposures to toxicants (Cairns et al., 1986).

2. *Colpidium campylum* or *Uronema parducz* grown for 19 to 43 h in culture with *E. coli* for food. The ciliate is exposed to several concentrations of toxicant to determine the minimum active dose affecting density (Dive and Leclerc, 1975; Sloof et al., 1983).

3. *Entosiphon sulcatum* grown in the presence of *E. coli* and toxicant for 72 to 96 h and density determined with a cell-counter (Bringmann and Kuhn, 1980).

3.3 Lethality

1. *Brachionus* mortality. This assay involves the reconstitution of rotifer (*Brachionus*) cysts before exposure to toxicants. Mortality is recorded after 24 h (Burnett, pers. comm.).
2. Use of sessile ciliates which are exposed to toxicants in petri dishes. Counting is time-consuming and accuracy is limited (Dive and Leclerc, 1975; Sloof et al., 1983).

3.4 Motility

Testing of the effects of toxicants on the motility of *Paramecium* (in Dive and Leclerc, 1975).

4.0 Bacterial Tests

4.1 Bacterial Enumeration Techniques

Although several tests in this section are not toxicity tests *per se*, they are used in battery approaches as indications of fecal contamination, and have been compared to other microassay tests.

1. Colony Counts: performed for *Legionella* (Dutka et al., 1983) in 24 to 72 h, but this species is very difficult to isolate from water due to its very low density, and isolation techniques are such that less than 10% of the population is recovered during estimation techniques.

2. Most Probable Number (MPN) Technique: involves the use of multiple fermentation tubes which are inoculated with serial dilutions of the culture to determine density.

   - This technique is performed for *Clostridium perfringens* in 24 to 48 h (Dutka et al., 1986) and gives an indication of present sewage contamination (Dutka, 1988). The test can be done on refrigerated samples after 36 h storage without changing the estimate, and since

spore formation only occurs in the intestine of warm-blooded animals, the presence of spores can be an indication of past fecal pollution activities at the site (Dutka and Rao, 1988).

- This technique is also used for *E. coli* through a membrane filtration technique using an *E. coli*-specific agar (MTEC) with membranes transferred to pads saturated with urea substrate. The main problem with these estimates is that the density estimates can be anywhere from 6 to 90% of the potential population, depending on the degree of stress imposed on the bacteria (Dutka et al., 1986). Presumably, bacteria which are stressed are not as hardy, and consequently, can not be separated accurately. *E. coli* can be difficult to separate from *Klebsiella* and *Enterobacter* spp..

- Fecal coliforms are isolated using a five tube, three to four decimal series MPN procedure using A1 broth. The A1 MPN procedure is superior to the FCMF (fecal coliform membrane filtration) technique, but in either case, estimates may only represent as little as 40% of true numbers when dealing with a stressed bacterial population (Dutka et al., 1986; Sim and Dutka, 1987).

3. Metabolic Indicators of Bacterial Presence

- Fecal sterol levels correlate with *E. coli* levels (Dutka et al., 1986) and consist of the measurements of cholesterol and coprostanol, the principle sterol metabolite in mammalian faeces (transformed by bacteria in the lower intestine). Cholesterol is not as specific an indicator as coprostanol, since the only known source of coprostanol is mammalian faeces. Sediment levels of coprostanol are a good indicator of mammalian fecal contamination. However, recent studies have found inconsistent correlations of sterol levels with other bacteriological data, and the test is no longer considered amenable to one-time, random sample collection (Dutka et al., 1988a).

- Coliphage Test: coliphage are bacterial viruses which replicate in lactose-fermenting Enterobacteriaceae. The APHA method counts viral plaques which form on a lawn of *E. coli* colonies after 6 or 24 h (Sim and Dutka, 1987). Positive findings suggest the presence of pathogenic viruses and fecal coliforms (Dutka and Rao, 1988), and the test is used to estimate the efficiency of sewage plants to remove human pathogenic viruses (Dutka, 1988). Turbidity may decrease coliform estimates up to 90% due to absorption (Dutka et al., 1986).

- Dehydrogenase Activity: the transport of electrons through cellular electron transport systems can be short-circuited by the use of redox dyes such as resazurin and TTC. These compounds are preferentially reduced by the electrons (i.e., in the place of oxygen). The reduction process alters the spectoral properties of the dye and results in a colour change which can be measured and related back to overall metabolic activity.

- Oxygen Consumption: used by Busch (1982), and could be used in a battery approach as a simple indicator of contamination analogous to the use of conductivity proposed by Kaiser (pers. comm). To be useful, the test requires standardization, since most of the variability can be attributed to differences in the bacterial population (Dutka and Kwan, 1984).

- Epifluorescence: Delesmotte and Delattre (1983) conducted an estimation of the epifluorescence of bacterial communities for 8 h before formal fixation and identification of the bacterial species. Although details were not specific, it is assumed that identification must have been restricted to gram positive and gram negative types. This technique would be very inaccurate.

4.2 Growth Inhibition by Toxicants

1. Estimation of Turbidity: is used as a numerical estimate of bacterial density. Measurements of the impacts of toxicants on colony growth have been identified for *Pseudomonas fluorescens* (Dutka and Kwan, 1981; Trevors et al., 1981, 1983), *P. putida* (Slabbert and Grabow, 1986), *Aeromonas hydrophila* (Dutka and Kwan, 1982), *Clostridium perfringens* (Dutka et al., 1986; Dutka et al., 1988a) and *E. coli* (Dutka et al., 1986). Techniques are variable, but include the exposure of 25 mL of log phase culture to 25 mL of the toxicant for 18 h (Dutka and Kwan, 1984), with the subsequent determination of changes in optical density by spectrophotometry (Dutka and Kwan, 1981, 1982; Trevors et al., 1983), nephalometry (Robinson, 1988), or determination of the toxicant concentration required to decrease growth in serial dilutions (Slabbert and Grabow, 1986). The technique is portable and basic, but each species has its own pattern of sensitivity to toxicants. The coefficients of variation for sewage effluents have ranged from 9 to 16 % (Robinson, 1988). Slabbert and Grabow (1986) found their estimate of toxicant concentration required to decrease growth in serial dilutions to be equal to the ability of *Tetrahymena pyriformis* motility estimates to document impact.

2. Prevention of Growth: ECHA Biocide Monitor is a new dipstick test which has received considerable support from Dutka (pers. comm.). The test involves plexiglass dipsticks (which should be available commercially soon) which contain a small absorbent pad impregnated with *Bacillus* bacteria. Growth of the bacteria is stimulated by wetting of the dipstick for approximately 10 sec, after which the excess fluid is drained away, and the dipstick is incubated at 35 to 37°C for 18 to 24 h (Dutka and Gorrie, 1989). Successful bacterial growth turns the pad inside the dipstick red. The presence of high levels of toxicants prevents (or reduces) bacterial growth on the pads, and a colour card may be available for interpretation.

3. Agar Diffusion Technique: a pure strain of *E. coli* (i.e., NTC10418) is inoculated in a plate of agar, and the toxicant is applied to the agar either in wells which are cut into the surface of the agar or on paper discs (Thompson and Watling, 1985). To apply on paper discs, the toxicant

sample is evaporated to near-dryness, dissolved in a known volume of water and a subsample (100 µl) is added to the paper disc before application to the agar. After incubation for 18 to 20 h, the zone of inhibition around the discs (or wells) is documented.

The use of 6 or 7 different concentrations allows a calculation of the minimum concentration required to produce a clear spot on the agar. The technique is best used for quality-control screening of metal-rich effluents over a prolonged period of time (Thompson and Watling, 1985), or for pre-screening for biodegradability using mixed bacterial cultures (Liu and Kwasniewska, 1981). The agar plates have a shelf life of 6 week at 4°C if kept in a sealed plastic bag, but the plates must be pre-dried before seeding (Liu and Kwasniewska, 1981).

4. Spore Germination Test: using *Bacillus subtilis* to calculate an EC50 (Yasuda et al., 1982).

5. ATP levels:

- ATP-TOX Test: usually uses *E. coli* because of its sensitivity, but any bacterial species with a short duplication period may be utilized, including *P. fluoresces* and *Salmonella typhimurium* TA98 (Ames test bacterium). Each species has its own sensitivity spectrum. The test measures the inhibition of bacterial growth and luciferase activity (Xu and Dutka, 1987a,b; Dutka et al., 1988a,b) and is usually the most sensitive microassay when batteries of tests are used. This sensitivity is attributed to the fact that a positive result is recorded at 1% inhibition, whereas other tests usually rely on a change in the EC50 (50% inhibition).

  The test relies on the assumption that the concentration of ATP per bacterium is constant and that total ATP concentration can be used to estimate bacterial density. This assumption may not always be valid. In the ATP-TOX test, ATP estimates are recorded at 1.2, 3.5 and 5 h, but can be extended to 7 or 8 h if necessary. Test durations of less than 5 h are preferred due to the possible accumulation of by-products associated with bacterial

growth or death, or the biodegradation of the toxicant. The test requires a Turner Designs Model TD-20e Luminometer. Relationships with other bioassays are described in Section 4.2.

- ATP in Activated Sludge: 1 mL of sludge is added to 35 mL of boiling TRIS for 30 to 60 sec, put into an ice bath, diluted and luciferase (firefly enzyme) is added before measurement of fluorescence (Levin et al., 1975).

4.3 Impact on Bacterial Metabolism (Fluorescence, Motility, Respiration, Enzyme Activity)

1. Fluorescence:

- Microtox: uses a marine bacterium (*Photobacterium phosphoreum*) which has a natural fluorescence, and the assay monitors the impacts of contaminants on the amount of fluorescence. The bacteria are available in a freeze-dried state, and can be reconstituted in the field. This test is the most widely accepted and applied microassay, and is discussed in detail in Section 4.1. In general, advantages of this test are that it is among the quickest available, the test is fully applicable to the field, and all reagents are available commercially. Disadvantages are that start-up requires some initial expense, the reaction requires some stabilization time and there may still be some problems with pH adjustment, timing of the reaction, salinity, coloured or turbid samples, and extraction or concentration of the toxicant in field samples.

- Dark mutant (*P. phosphoreum*): This test uses a mutant form of the Microtox bacterium which does not emit light. Exposure to mutagenic contaminants results in a black-mutation to its original form, which results in the emission of light. This test is very recent (Dutka, pers. comm.) and will not be available commercially for some time yet. It is thought to be more sensitive and accurate than the current Microtox test.

- Microtox with *Beneckea* (*Lucibacterium*) *harveyi*: not predictive of *Daphnia*, rainbow trout or fathead minnow bioassays for organics (Shiotsuka et al., 1980).

- Tchan: involves the determination of the fluorescence of a mixture of bacteria and algae. The test uses *P. phosphoreum* (NZIID) similar to Microtox, but includes a coincubation with an algal species *Dunaliella tertiolecta* (McFeters et al., 1983). A field photobioluminator was constructed and the test gives a 1 or 24hEC50, with the 24 h EC50 being approximately 44% lower.

- Crude Luciferase Extracts: extracts from the firefly (*Photonius pyralis*) or *Vibrio* (*Photobacterium*) *fischeri*, can be used with a portable, monolight luminescence photometer (Surrowitz et al., 1987). The necessary reagents are added to either the firefly preparation (ATP) or the bacterial extract (NADH, FMN, and n-decaldehyde); both luciferase preparations are available commercially. These tests are more sensitive than Microtox to inorganic toxicants (Cd, Zn, Cu) (Surowitz et al., 1987).

2. Motility: subjective estimates of motility for the flagellated bacterium *Spirillum volutans* are used to assess toxicant impact. The concentration of toxicant (or effluent) which is required to decrease 90% of the motility gives an estimate of the MEC90. Accurate measurements require a culture in log growth phase and estimates are restricted to cultures which show >90% motility in control groups, as well as frequent, quick changes in direction (Qureshi et al., 1982). The assay can give an indication of low levels of specific toxicants affecting flagellar coordination (enzyme inhibition or neurotoxic effects). In inter-assay comparisons, this assay is usually the least sensitive microassay (Dutka and Kwan, 1982; Dutka et al., 1983), except in some assays of sediments contaminated with complex organic chemicals (Dutka et al., 1988a,b). The technique is not easily applied to field situations. The life span of the culture is very short (3 to 5 d), the cultures must be transferred every 24 h and the organism is very sensitive to movement and stress (Dutka, 1986). Current estimators use relatively complex video equipment to observe the bacteria over a period of time

from 5 to 120 min (Dutka and Kwan, 1982, Dutka et al., 1983; Coleman and Qureshi, 1985) with the 120 min observations showing much greater sensitivity (Walker, 1988).

3. Nitrification:

- *Nitrobacter* is a strict aerobic bacterium which obtains its energy from the oxidation of nitrite to nitrate. The nitrification test involves the resuspension of freeze-dried bacteria in the toxicant for 4 h and measurement of the disappearance of nitrite (colourimetrically) or the production of nitrate (ion-specific electrode) (Williamson and Johnson, 1981). The bacteria show about 30% viability on reconstitution, and sample measurements are made every 30 min for 4 h by removal of a subsample for direct measurement. The coefficient of variation when used on sewage effluents is 10 to 12 % (Robinson, 1988).

- Activated sludge: measurement of nitrification inhibition (Robinson, 1988). Washed activated sludge from nitrifying treatment are added at 3000 mg solids/L to 125 mL domestic sewage, 25 mL nutrient medium and 100 mL test material. The oxidized nitrogen produced, or ammoniacal nitrogen removed in 30 min or 3 h is recorded relative to a control. A commercially produced culture (BIOCYTE) is available to standardize the test (Robinson, 1988).

- Nitrification rate of *Azotobacter vinelandii*: grown in nitrogen-free media for 18 h, resuspended and put in a sealed flask with toxicant under acetylene (Tam and Trevors, 1981). The mixture is incubated at 30°C for 12 h and gas is removed at time intervals for assay of ethylene (nitrogen-fixation estimate), oxygen and carbon dioxide.

4. CO_2 production: potentiometric determinations of the CO_2 production of *E. coli* cells which are immobilized at the surface of a CO2-sensing electrode (Dorward and Barisas, 1984). Bacteria must be harvested at the maximum growth stage at 37°C and used within 40 h. The assay response time

is very fast (3 to 20 min), but requires some expertise. Problems associated with measurements include the interference of some test substances with the CO_2 electrode, such as organic acids, dissolved gases, sodium hypochlorite, nitrite, arsenite and phenol (Dorward and Barisas, 1984).

5. Oxygen Consumption:

- *Pseudomonas putida* respiration measured after 10 min or 7 h incubation (Slabbert and Grabow, 1986). Although the 7 h incubation is more sensitive, the results are qualitatively the same and results are directly comparable to those obtained from the growth of *P. putida* during exposure to industrial effluents and laboratory mixtures. The assay is highly sensitive to Cu, Cd and Zn (more than Microtox) but is insensitive to cyanide, phenol and Hg (Slabbert and Grabow, 1986).

- Biological oxygen demand of activated sludge is very useful for monitoring the potential impacts of chemicals and waste streams on biological treatment plants. The OECD (Organization for Economic Development and Cooperation) has added this test to their list of tests for screening chemical toxicity. Activated sludge at 4000 mg solids/L is mixed with a synthetic sewage concentrate and is aerated along with the test solution (Robinson, 1988) for 30 min (Elnabarawy, 1986) or 3 h (Brown et al., 1981; Dutka et al., 1983). The test equates toxicity with the inhibition of microbial respiration and measures the concentration of chemicals required to decrease the oxygen content by 50% relative to the control group. Also used by Bauer et al. (1981) to compare the time required to deplete 50% of the oxygen in toxicants relative to a control group. The test requires aeration and the use of an oxygen electrode.

  The main disadvantage of this technique is that inter-laboratory variability yields a coefficient of variation which ranges from 83 to 108% for inorganic and organic chemicals (Cr^{+6}, cyanide, formaldehyde and toluene) (Elnabarawy, 1986; Elnabarawy et al., 1988). The

within-batch coefficient of variation is 9%, but between batches can be as high as 28 to 76% (King and Painter, 1986), which leads to inconclusive results when comparing between laboratories or sampling dates.

- Dissolved oxygen depletion is measured with the use of an oxygen electrode every 30 min for 3h during incubation with activated sludge at 1 control and 4 to 7 toxicant concentrations (Dutka and Kwan, 1984; Greene et al., 1985). A synthetic activated sludge can be constructed from bacterial colonies isolated from different sludges in an attempt to standardize bacterial colonies. The ranges of coefficients of variability have been reported as 7 to 11% (Robinson, 1988) and 15.7 to 25.1% (Dutka and Kwan, 1984; Retcuna et al., 1986). The technique is insensitive to copper (Reteuna et al., 1986).

- Polytox represents a commercially available specialized mixture of bacterial cultures (Elnabarawy et al., 1988) which is rehydrated in aerated distilled water in BOD bottles along with the chemical test solutions. Dissolved oxygen is measured at 20 and 30 min, and the oxygen consumption is compared to controls. The major advantage of this technique is that it provides a common substrate for inter-laboratory comparisons and the process can be regarded as a pre-screening tool for the activated sludge process.

6. ATP levels: are in activated sludge by techniques used for algae or the techniques described for ATP-TOX.

7. Glucose uptake by activated sludge: glucose is added to a continuously aerated mixture of activated sludge, and glucose utilization is measured with a glucose-sensitive electrode to calculate the time required for a 50% decrease in glucose levels (Olah and Princz, 1986). The reaction is very sensitive to low oxygen levels, and the sensitivity of the assay for detecting toxicants is intermediate between protease enzyme activity (more sensitive than glucose) and respiration (less sensitive) (Olah and Princz, 1986).

8. Enzyme activity.

- Beta-galactosidase.

Toxichrome test: a test developed which may eventually replace the Microtox test (Dutka, pers. comm.). The test uses a strain of *E. coli* which have been freeze-dried. Reconstitution of the bacteria initiates a turning-on of enzyme systems, and the "start-up" of the beta-galactosidase enzyme system normally requires about 40 min to reach full activity. This rate of enzyme "turn-on" and the maximal activity reached are both affected by the presence of toxicants. The monitoring of beta-galactosidase activity over a 2 h period allows assessment of toxicant impact, and results are read with a spectrophotometer. Portable readers and interpretation cards are available for field use (Vigerstad, pers. comm.).

SOS-Chromotest: a new genotoxicity test described by Dutka et al. (1986) and Xu et al. (1987). This assay also uses colorimetric indicators for activity of the beta-galactosidase (BGSD) enzyme system as an indicator of toxicant impact. The strain of *E. coli* used (K12-PQ37) has been genetically altered so that the galactosidase enzyme acts as an indicator of activity of cellular DNA repair mechanisms. The SOS-repair system is induced after DNA damage, and in this strain of bacteria, one of the control genes for the SOS system (sfia) has been fused to an operon which acts to turn-on the BGSD enzyme (lac-z) when the SOS-system is activated (Quillardet et al., 1982). The test therefore provides an indication of chemical pollution which may have genotoxic or mutagenic effects (Dutka, 1988), and the BGSD activity acts as a marker for genotoxic impacts. To distinguish between toxicants which kill the bacteria and those causing DNA damage, the assay also incorporates simultaneous determinations of alkaline phosphatase (AP) activity. Positive recording of AP activity ensures that the bacteria are still viable during the test.

The assay measures the SOS-inducing potential and can be used with rat liver microsomal mixtures (S9) similar to the Ames test. The S9 mixture is used to simulate the liver metabolic capabilities of mammals, and the microsomal fraction contains enzymes which convert the toxicants into forms which would be found in mammalian systems after exposure to the metabolic pathways in mammalian livers.

- Dehydrogenase activity: a measure of heterotrophic potential. Dehydrogenases are an enzyme class which is a major representative of the enzymes which catalyze oxidation reactions by transferring electrons down the electron transport system. During incubations (generally less than 90 min), a redox-indicator dye is added to produce a colour changes (Elnabarawy, 1986). Various dyes have been used (reviewed in Bitton, 1983; Bitton et al., 1986), including methylene blue, triphenyl tetrazolium chloride (TTC), tetrazolium blue, resazurin and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT).

  Resazurin reduction: used to measure dehydrogenase activity of *Bacillus cereus* (Liu, 1985, 1986). Reagents are commercially available, and bacteria can be used from laboratory cultures or they can be isolated from the environment. Each bacterial species has its own sensitivity spectrum, and in general, the gram positive bacteria are more sensitive than the gram negatives (Liu, 1986). Liu (1985) used 6 different bacterial cultures and found that they all responded differently and that the dose response was not linear for most chemicals. Elnabarawy (1986) used activated sludge to measure resazurin reduction.

  The reaction is run at 21°C for 20 to 30 min and requires a spectrophotometer. The reaction is very stable, reproducible, can be performed within 2 h and has a coefficient of variation of 3% (Liu, 1986). The test has been validated for As^{+5} and $HgCl^2$ (Liu, 1985).

TTC reduction: used to assess the dehydrogenase activity of activated sludge. The mixture is incubated at 37°C, pH 7.5 for 60 min and the results are read on a spectrophotometer. TTC is sensitive to cyanide (Slabber and Grabow, 1986).

INT Activity: *Pseudomonas alcaligenes* exposed to toxicant over a range of 4 to 5 concentrations at 28°C for 30 min with phosphate buffer (Walker, 1988). DMSO, ethanol and acetone have been used as solvents at concentrations of 3 to 6%, although enzyme activity in DMSO is generally less than in ethanol or acetone, and the assay is sensitive to pre-incubation and diluent type (Burton and Lanza, 1985; Walker, 1988).

- Glucose-6-phosphate dehydrogenase (type XV from Baker's yeast). A commercial kit is available (Mallak and Brunker, 1984) which uses spectrophotometric evaluation of the reduction of ion absorbance after an exposure of 5 to 45 min.

- Histidine synthesis: the Ames test uses special strains of *Salmonella tryphimurium* (TA 1535/1537) which have been altered such that they cannot grow in the absence of histidine. The test involves the measurement of the potential of toxicants to induce a back-mutation to the parental form, which can grow in the absence of histidine (Ames et al., 1975; Maron and Ames, 1983). The assay requires incubation for 48 to 72 h, and the number of revertants are counted. Pre-incubation treatment with and without liver enzyme (S9) preparations simulate mammalian metabolic capabilities. The test can also be used for sediment extracts (Klekowski and Levin, 1979).

5. Invertebrate Assays

Invertebrate assays: this listing is not intended to be complete, but presents a brief summary of tests encountered during the literature review which were compared with microassays or have been reported to

have some potential as field monitoring tests.

5.1 *Daphnia magna* Acute Lethality Test

Daphnia magna: details are available in several sources, including Poirier et al. (1988). The test is commonly used as a 48 h bioassay to determine the concentrations of toxicants required for immobility (EC50) or lethality (LC50). For rapid screening procedures, the test is also used as a 24 h test.

5.2 Sea Urchin Early Embryo Growth Test

Sea urchin early embryo growth test: a marine test which uses any of a number of sea urchin species, and is available in several forms.

1. ^{3}H-thymidine incorporation is described for *Arbacia punctulata* by Nacci et al. (1986). The test is relatively rapid, yielding results in less than 5 h and requires less than 100 mL of sample. However, the test assesses toxicity by looking for changes in ^{3}H-thymidine incorporation in 4 h old embryos. Samples are exposed for 2 h before addition of radio-labelled thymidine and exposure is continued for 2 h. The test is not applicable to field testing because of the use of radioactive material and requires special handling equipment.

2. Developmental bioassay is described for *Strongylocentrotus purpuratus* by Hose (1985). Gametes are exposed to toxicants before fertilization and embryos are allowed to develop for 48 h at which time survival and teratogenesis are evaluated. Embryo mitotic rates are used as indicators of the overall health and genotoxicity is assessed by examining stained embryos at the light microscopy level. This assay is too complex and slow for full consideration as a field microassay.

5.3 Sea Urchin Sperm Toxicity Test

Sea urchin sperm toxicity testing is described for *Arbacia punctulata* by Nacci et al. (1986). This test provides data within 5 h and requires less than 100 mL of sample. Gametes are collected by electro-stimulation, spermatozoa are diluted to a constant density and the toxicant is added for 1h at 20°C. Eggs are then added and fertilization rates are determined by observing the presence/absence of fertilization membranes 20 min after adding the eggs. This test is quick, efficient and requires no extraordinary procedures or equipment, but does require reproductively active sea urchins. The disadvantages are that some chemicals are extremely toxic to spermatozoa, and the toxicity of these compounds could be overestimated.

5.4 Copepod Toxicity Test

Copepod toxicity test: a 96 h LC50 test described by Tarkpea et al. (1986) for *Nitocra spinipes*. The test is used routinely for pollution monitoring in Swedish laboratories.

5.5 Bivalve Embryo Toxicity Test

Bivalve embryo toxicity test: a 48 h test examining the impacts of sediments on the embryos of the oyster, *Crassostrea gigas* or mussel, *Mytilus edulis* (Williams et al., 1986b). The test adds sediments and seawater to fertilized oyster eggs, but it is difficult to get gravid female oysters during some seasons, especially spring and winter.

5.6 Amphipod 10 d Survival Test

Amphipod 10 d survival test: requires field collection of specimens before exposure of amphipods to sediments in clean seawater (Williams et al., 1986b).

5.7 Chironomid growth reduction

Chiromonid growth reduction using *Chironomus tentans* exposed to toxicants for 10 d (Giesy et al., 1988).

5.8 Nematode Mutagenicity/Lethality Test

Nematode mutagenicity/lethality test: using *Panagrellus redivivus* as described by Samoiloff et al. (1983), Thomas et al. (1986) and Samoiloff (1989). Synchronous larvae are collected by putting gravid females on non-nutritive agar for 12 h and collecting the juveniles. The juveniles are grown in test agar for 96 h. The test gives mutagenicity results which are comparable to the Ames test, but is much less expensive.

5.9 Chronic/Acute Lethality Bioassays

Bioassays using a variety of species of invertebrates, including shrimp, (*Crangon septemspinosa)* (Ribo and Kaiser, 1983), *Daphnia magna* (Poirier et al., 1988), *Ceriodaphnia* (Mount and Norberg, 1984), *Hyallela azteca* or various insect species (Walker, 1988).

6. Vertebrate Tests

Vertebrate tests: no references are given, and the list is not intended to be complete. The following tests represent the majority of vertebrate tests compared with microassays and full details are available elsewhere.

6.1 Acute Lethality Bioassay

Acute lethality bioassay: 96 h bioassays are completed following standardized procedures for a wide variety of species, including rainbow trout (*Salmo gairdneri*), fathead minnow (*Pimephales promelas*), sunfish (*Lepomis macrochirus*), brown trout (*Salmo trutta*), sheepshead (*Cyprinodon variegatus*), golden orfe

(*Leuciscus idus melanonus*) and guppy (*Peocilia reticulata*) and fish are exposed in continuous-flow or static systems or are exposed by intra-peritoneal (i.p.) injection.

6.2 Residual Oxygen Demand Test

Residual oxygen demand test: fish are exposed to the toxicant in sealed vessels and the residual oxygen content of the water is measured at death. As the concentration of toxicant increases, the amount of oxygen remaining in the flask at death increases and an EC50 is calculated as the concentration required to kill a fish and leave 50% of the available oxygen in the flask. The test is very rapid, taking less than 8 h, and in effluent tests was not significantly different from the 96 h LC50 for rainbow trout 12 out of 13 times (Atwater et al., 1983). Comparability with the *Daphnia* test is much lower (r= 0.42) (Atwater et al., 1983).

APPENDIX B

LIST OF PERSONAL COMMUNICATIONS

Listing of the scientists cited as pers. comm. or contacted during this review.

Blaise, C., E.P.S., Longueil, Quebec	514-651-6862
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Scroggins, R., E.P.S., Hull, Quebec	819-997-1223
Vigerstad, T., Orgenics Distributor, Bethesda, Maryland	301-881-5383